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Influence of microplastics on the toxicity of cephalexin to early juveniles of the common goby (*Pomatoschistus microps*) in relation to temperature change

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Influence of microplastics on the toxicity of cephalexin to early juveniles of the common goby (*Pomatoschistus microps*) in relation to temperature change

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Abstract

In the last decades, the contamination of the marine environment by pharmaceuticals and microplastics, which are considered emerging contaminants of high concern, has been increasingly attracting the attention of the scientific community, stakeholders, and the citizens in general. More knowledge is needed to improve the basis for their human and environmental risk assessments under the current scenario of global warming.

The main goals of the present study were to investigate the toxic effects of cephalexin, alone and in combination with microplastics, on wild juveniles of the common goby (*Pomatoschistus microps)* and if a 5ºC raise of water temperature modulates the chemically induced toxicity. Three hypotheses were tested: (i) water concentrations of cephalexin in the low ppm range are toxic to *P. microps* juveniles; (ii) the presence of microplastics in the water influence the toxicity of cephalexin to the test organism; and (iii) the 5ºC raise of temperature (20ºC - 25ºC) modulate the effects of the tested substances.

Cephalexin was selected as test substance because is a widely used antibiotic that has been found in the environment. Polyethylene microspheres (MP) (1-5 µm diameter) were used as MP model because polyethylene is one on the most used plastic polymers and is among the most found in the marine environment and biota. *P. microps* was selected as model species for this study mainly because is a common species in several European estuaries and other coastal ecosystems, where it plays a major role as an intermediate predator, being a crucial prey for several species, including some included in the human diet.

The experimental work was divided in four main phases. The first phase consisted in getting training and validates the methodologies for further use during the experimental work. In a second phase, one spectrophotometry and one spectrofluorometry methods allowing the determination of the actual concentrations of cephalexin and MP, respectively, in test media during the bioassays, were adapted to artificial sea water (ASW) and validated. Briefly, a series of cephalexin nominal concentrations were prepared in ASW, and their absorbance was read at 260 nm. Absorbance data were plotted against the respective cephalexin nominal concentrations and a linear regression model was fitted to the data. A similar procedure was used for the MP calibration curve but using fluorescence (470/588 nm, excitation/emission wavelengths) instead of absorbance. In the third phase of the study, a preliminary toxicity bioassay to find the range of cephalexin concentrations inducing toxic effects on *P. microps* juveniles was carried out. In the range of concentrations tested (0.313 – 5 mg/l), the effects of cephalexin were not clear. Thus, it was decided to test a range including a highest

cephalexin concentration in the final bioassays. In the final phase of the experimental work, the effects of cephalexin alone (1.25 – 10 mg/l) and in the presence of MP (0.184 mg/l) on *P. microps* juveniles were investigated at 20ºC and 25ºC. The bioassays were carried out for 96 h, with 12 fish exposed individually per treatment at each temperature. Criteria indicative of toxicity were: the post-exposure predatory performance (hereafter indicated as predatory performance), the activity of the enzymes acetylcholinesterase (AChE), glutathione S-transferases (GST) and ethoxyresorufin-O-deethylase (EROD), and lipid peroxidation levels (LPO). The concentrations of cephalexin and MP and the decay of both substances along the exposure period were determined. When tested alone, cephalexin significantly decreased the predatory performance of the fish (≥ 2.5 mg/l) and induced AChE and GST activity (1.25 and 10 mg/l, respectively). These findings indicate that cephalexin is able to induce toxic effects on *P. microps* juveniles at concentrations in the low ppm range, thus corroborating our first hypothesis. At 20ºC, fish simultaneously exposed to cephalexin and MP, had inhibition of the predatory performance (≥ 1.25 mg/l) and increased AChE (≥ 5 mg/l) and GST activities (≥ 1.25 mg/l). Therefore, the presence of MP influences the toxic effects of cephalexin, corroborating our second hypothesis. The comparison of the control groups at 20ºC and 25ºC shows that the increase of temperature changed some of the analyzed parameters. A significant interaction between treatments and temperature was found for all the parameters assessed, suggesting that temperature modulates the effects of the tested substances, thus corroborating our third hypothesis.

In summary, the results of the present Thesis indicated that cephalexin is able to cause adverse effects on *P. microps* juveniles health after short-term exposure through the water to concentrations in the low ppm range, that the presence of MP in the water influences the cephalexin-induced toxicity to these organisms, and that the raise of temperature (20ºC to 25ºC) increased some of the toxic effects of cephalexin and MP. These findings highlight the need of more research on the combined effects of mixtures of emerging contaminants to increase the basis for the risk assessment of these substances.

Keywords: *Pomatoschistus microps*, cephalexin, microplastics, temperature & global warming, multi-stressors effects, biomarkers, predatory performance.

Resumo

Nas últimas décadas, a contaminação dos ecossistemas marinhos por fármacos e microplásticos, considerados contaminantes emergentes de elevada preocupação, têm vindo a atrair a atenção da comunidade científica e dos cidadãos em geral. É necessário aumentar o conhecimento sobre os efeitos tóxicos destas substâncias para uma melhor avaliação dos riscos ambientais e humanos no atual cenário de alterações climáticas.

Os objetivos principais do presente estudo foram investigar os efeitos tóxicos do antibiótico cefalexina em juvenis do góbio comum (*Pomatoschistus microps*), se a presença de microplásticos influencia a toxicidade da cefalexina para os organismos testados e se um aumento da temperatura da água modula a toxicidade das substâncias testadas. Foram testadas três hipóteses: (i) as concentrações de cefalexina em água do mar artificial (ASW) na ordem dos ppm são tóxicas para juvenis de *P. microps*; (ii) a presença de microplásticos na água influencia a toxicidade da cefalexina nos organismos testados; e (iii) um aumento de 5ºC da temperatura da água (20ºC - 25ºC) modula os efeitos das substâncias testadas.

A cefalexina foi selecionada como substância teste por ser amplamente usada e encontrada no meio ambiente. Por sua vez, as microesferas de polietileno (MP) (1-5 µm de diâmetro) foram usadas como modelo de microplásticos, porque o polietileno é um dos polímeros de plástico mais usado e encontrado no meio ambiente. *P. microps* foi a espécie escolhida como modelo para este estudo por ser abundante em vários estuários da Europa e outros ecossistemas costeiros, onde desempenha uma função ecológica relevante enquanto predador intermédio, sendo uma presa importante para várias espécies, incluindo algumas de consumo humano.

O trabalho experimental foi dividido em quatro fases. Na primeira fase foram adaptadas e validadas as metodologias para realizar o trabalho experimental. Numa segunda fase, um método espectrofotométrico e um método espectrofluorimétrico para posterior determinação das concentrações reais de cefalexina e MP em água do mar artificial (ASW) foram adaptados e validados. Resumidamente, prepararam-se uma série de soluções em série com diferentes concentrações de cefalexina em ASW, sendo a absorvância de cada uma lida a 260 nm. Foi ajustado um modelo de regressão linear aos dados. Usou-se um procedimento similar ao anterior para realizar uma curva de calibração de MP, mas usando fluorescência (470/588 nm, comprimento de onda de excitação e emissão) em vez de absorvância. Na terceira fase do estudo, efetuou-se uma avaliação preliminar da toxicidade da cefalexina em juvenis de *P. microps*. No intervalo de concentrações testadas (0.313 – 5 mg/l), os efeitos da cefalexina não foram muito claros, pelo que posteriormente se testou também uma concentração mais elevada de

cefalexina. Na fase final do trabalho experimental, foram avaliados os efeitos resultantes de uma exposição à cefalexina (1.25 – 10 mg/l) e de uma exposição simultânea a este antibiótico e a MP (0.184 mg/l) a 20ºC e a 25ºC. A duração dos bioensaios foi de 96 h. Para cada temperatura, foram utilizados 12 peixes por tratamento (expostos individualmente). Os critérios indicativos de toxicidade foram: a performance predatória após exposição (doravante indicada por performance predatória), a atividade das enzimas acetilcolinesterase (AChE), glutationa S-transferase (GST) e etoxiresorufina-O-desetilase (EROD), e os níveis de peroxidação lipídica (LPO). Determinaram-se ainda as concentrações reais de cefalexina e MP no meio de teste e o seu decaimento ao longo do período de exposição. Nos peixes expostos apenas à cefalexina, observou-se uma diminuição da performance predatória (≥ 2,5 mg/l) e uma indução da atividade das enzimas AChE e GST (1,25 e 10 mg/l, respetivamente). Estes resultados indicam que a cefalexina induziu efeitos tóxicos em *P. microps* em concentrações na ordem ppm, corroborando assim a nossa primeira hipótese. A 20ºC, os peixes expostos simultaneamente à cefalexina e MP, apresentavam uma performance predatória reduzida (≥ 1,25 mg/l) e um aumento da atividade da AChE (≥ 5 mg/l) e GST (≥ 1.25 mg/l). Estes resultados indicam que a presença de MP influenciou a toxicidade da cefalexina, corroborando assim a nossa segunda hipótese. A comparação dos grupos controlo dos ensaios a 20ºC e 25ºC mostra que o aumento de temperatura modifica alguns parâmetros estudados. Todos os parâmetros testados demonstraram ter interações significativas entre tratamentos e temperatura, sugerindo que a temperatura modula os efeitos das substâncias testadas, corroborando a nossa terceira hipótese.

Resumindo, os resultados do presente estudo indicam que a cefalexina pode afetar a saúde dos juvenis de *P. microps* a concentrações na ordem dos ppm, que a presença de MP na água influencia a toxicidade da cefalexina, e que o aumento de temperatura (20ºC a 25ºC) afeta alguns dos efeitos tóxicos da cefalexina e MP. Estes resultados destacam a necessidade de aumentar o conhecimento sobre os efeitos combinados de contaminantes emergentes para uma melhor avaliação dos riscos que estas substâncias representam.

Palavras-chave: *Pomatoschistus microps*, cefalexina, microplásticos, temperatura & aquecimento global, efeitos multi-stressores, biomarcadores, performance predatório.

List of Abbreviations

1-ANOVA – One factor Analysis of Variance

2-ANOVA – Two factor Analysis of Variance

AChE – Acetylcholinesterase

ANOVA – Analysis of Variance

ASW – Artificial Sea Water

ChE - Cholinesterase

CYP1A – Cytochrome P450 subfamily 1A

DTNB – 5,5'-dithiobis-(2-nitrobenzoic acid)

EC_{50} – Median effective concentration

EROD – Ethoxyresorufin-O-deethylase

F – Fluorescence

GST – Glutathione-*S*-transferase

LC_{50} – Median lethal concentration

LPO – Lipid peroxidation

MP - Polyethylene microsphere

OD – Optical Density

OECD – Organization for Economic Co-operation and Development

PSU – Practical salinity units

r – Pearson correlation coefficient

TBA - Thiobarbituric Acid

TBARS – Thiobarbituric Acid Reactive Substances

TCA – Trichloroacetic Acid

u.p – Ultra pure

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CHAPTER I

1. Introduction

As a result of the increase of human population and global industrialization, the environmental contamination by a high diversity of chemical substances has been raising over time (Gonçalves *et al.,* 2013). A considerable number of these substances, as well as some of the products resulting from their environmental fate, are able to induce adverse effects on human and ecosystem health at ecologically relevant concentrations (Ternes, 1998; Stuart *et al.,* 2014). Therefore, the risks that they pose to environmental and human health need to be assessed to improve the safety of their use. The environmental risk assessment (ERA) of chemicals is a process under continuous evolution, and more cost-effective methods are needed, especially to evaluate the risks of multi-stressors in complex ecosystems, such as estuaries and other coastal areas.

Estuaries are ecologically important ecosystems, where the fresh water from the river discharge rich in nutrients and organic matter and the entry of salt water, create unique conditions for a high number of species that spend their entire life cycle or particularly important parts of it (*e.g.* spawning, juvenile development) in these systems (Anacleto & Gomes, 2006). The conservation of estuaries is of great importance to ensure that there is preservation of marine ecosystems as a whole (Anacleto & Gomes, 2006; Gonçalves *et al.,* 2013).

In several regions around the world, estuaries are under a great pressure (Gonçalves *et al.,* 2013), resulting from diverse anthropogenic activities such as fishery, aquaculture, recreation activities, urbanization, diverse types of industry, among several others. These activities often result in lost and degradation of habitat resulting from territory occupation, physical barriers (*e.g*. bridges, tunnels, safety works, dams), pollution, over-exploration of resources, among several others. Therefore, estuarine conservation is often challenging.

1.1. Environmental contaminants in estuaries

Estuaries of impacted regions are contaminated by complex mixtures of several substances (Van der Oost *et al.,* 2003; Yu Chen Lin *et al.,* 2008; Fendall & Sewell, 2009; Andrady, 2011; Martins *et al.,* 2013; Oliveira *et al.,* 2013). In general, these include 'traditional' environmental contaminants that have been studied for a long time such as metals, pesticides, hydrocarbons, among several others, as well as the so called

'emerging' contaminants of high concern that started to be investigated relatively to their environmental effects more recently. A harmonized definition of 'emerging' contaminant does not exist yet but several were proposed so far (*e.g.* Boxall, 2012; United States Environmental Protection Agency (EPA)). The general concept is that they are substances that only recently received attention as environmental contaminants, not having yet specific regulations, for whose the knowledge on their environmental fate and adverse effects on the biota is still limited, and that the methods and approaches used to assess their environmental concentrations and/or toxic effects still need to be improved. 'Emerging' contaminants of high concern at the present include pharmaceuticals and personal care products, nanoparticles and nanomaterials, and microplastics, among several others, including substances resulting from their biotransformation (metabolites) and environmental fate (degradation products, aggregates, *etc*) (Ternes, 1998; Fendall & Sewell, 2009; Boxall, 2012). These substances have been increasingly found in environmental compartments and the biota at variable concentrations ranging from ppb or lower to low ppm ranges (Ternes, 1998). The simultaneous exposure of organisms to two or more environmental contaminants may result in toxicological interactions potentially inducing additive, synergistic, potentiation and/or antagonistic effects (Klaassen, 2008; Kortenkamp *et al.,* 2009; Monteiro & Boxall, 2009). From an environmental point of view, such interactions only recently started to be investigated and this is now considered a priority topic of research (Boxall, 2012).

1.2. Emerging contaminants in estuaries: pharmaceuticals

Pharmaceutical products are widely used with several of them being consumed everyday by a high number of persons and administered to animals and other organisms globally (Zenker *et al.,* 2014). Therefore, in several regions they are continuously found in the environment (Van der Oost *et al.,* 2003). In addition to their wide and intensive use (Laville *et al.,* 2004; Nikolaou *et al.,* 2007), the concerns regarding their presence in the environment are mainly because pharmaceuticals are biologically active substances able to induce adverse effects in exposed wild organisms and humans, as well as some of their metabolites and degradations products; several of these substances have a considerable environmental persistence, may accumulate in abiotic and biotic components of ecosystems; and their use is expected to increase following the increasing trend of human populations (Boxall, 2012; Martins *et al.,* 2013).

One of the most important classes of pharmaceuticals present in the environment is the antibiotics group. This class of pharmaceuticals is of particular concern because

several of them are able to induce the development of resistance and multi-resistance both in target species and no-target ones (Boxall, 2012). Antibiotics may also affect the environmental fate of other contaminants, including by slowing down their degradation rates (Monteiro & Boxall, 2009).

Among antibiotics, cephalexin ($C_{16}H_{17}N_3O_4S$) (Figure 1) has been considered of particular importance to study, mainly due to the wide and intensive use in Human and Veterinary Medicine (EMEA, 1999). It is a β-lactamic antibiotic, a first generation cephalosporin, derived from 7-aminocephalosporanic acid (Garrett *et al.,* 1994), that has a broad spectrum of activity against Gram positive and Gram negative bacteria (Garrett *et al.,* 1994). Cephalexin is used to treat several infections such as ear infections, respiratory and urinary tract infections, bone infections, among others (Cephalexin Information, 2014).

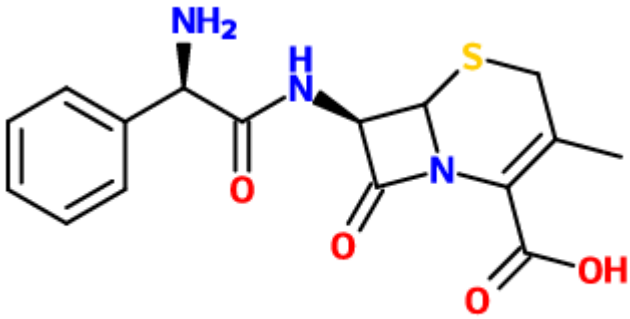


Figure 1: Molecular structure of cephalexin (adapted from Sigma-Aldrich, 2014).

The mechanism of cephalexin action is through the disruption of the peptidoglycan layer synthesis, a crucial process to maintain the cell wall structure (Sigma-Aldrich, 2014).

Cephalexin is administered orally, being absorbed through the digestive tract (Garrett *et al.,* 1994). The distribution of this substance in the organism is wide, diffuses into all the tissues except for the central nervous system, and crosses the placental barrier (Garrett *et al.,* 1994). Although it is mainly excreted through the urine (mainly in the parental form), bile excretion and breast milk excretion also occurs in small amounts (Garrett *et al.,* 1994). Human body half-life of cephalexin is 0.8-1.2 hours (Garrett *et al.,* 1994). Sigma-Aldrich Company (2012) provided toxicological information in the information sheet *Material Safety Data Sheet* (MSDS) where toxicological effects in rats when exposed to concentrations higher than 20000 mg/kg during acute toxicity tests are reported (oral administration). The oral median lethal dose (DL_{50}) of cephalexin to the

mouse, rat, guinea pig and rabbits is 1600 mg/kg, 3000 mg/kg, 1000 mg/kg, and 1000 mg/kg, respectively (EMEA, 1999).

Cephalexin has been detected in aquatic systems in different regions of the world. For example, in Asia, concentrations of 13 to 182 ng/l were found in Chinese saltwater surface waters (Gulkowska *et al.,* 2007), In Australia, a concentration of 2000 ng/l was found in wastewater, and the substance was found 500 meters from the place of discharge, revealing a poor treatment of effluents (Constanzo *et al.,* 2005). In Brazil, concentration of 242 ng/l was found in river surface water samples (Sodré *et al.,* 2010). In Taiwan, concentrations of 1563 - 4367 ng/l were found in waters entering wastewater treatment plant (Lin *et al.,* 2009).

The toxic effects of cephalexin on aquatic organisms were poorly investigated. The estimated cephalexin water concentration causing an effect of 50% (EC_{50}) on the duckweed (*Lemna gibba*) after a seven-day static renewal test, using wet weight, frond number, chlorophyll *a* and *b* and carotenoids was higher than 1000 mg/l (Brain *et al.,* 2004). The 96 h median lethal concentration (LC_{50}) of cephalexin to the freshwater planarian *Dugesia japonica* was also higher than 1000 mg/l (Li, 2013). A reduction of the number of new juveniles, an increase of dead juveniles and aborted eggs of *Daphnia magna* were observed at concentrations equal or higher than 125 mg/l after a chronic toxicity bioassay (Marques, 2011).

1.3. Emerging contaminants in estuaries: microplastics

Due to characteristics such as versatility, low cost, light weight and resistance, among others, plastics are used in a wide range of products and technology and their global production has been growing for more than 50 years. The global world production in 2012 rose 288 million tonnes (Plastics Europe, 2013). As the result of the wide production and use of plastics, considerable amounts of plastic debris enter into the environment after their manufacture and utilization (Barnes *et al.,* 2009). In the environment, such plastic fragments are “degraded” in a very slow rate and have been accumulating in the environment along decades, reaching now extraordinary concentrations in several aquatic systems, especially in ocean gyres causing problems to navigation, fisheries and other activities (Wright *et al.,* 2013). The first evidences of the adverse effects caused by plastics debris on wildlife were perhaps the physical effects induced in large sized species of mammals, birds, turtles, big fish, and other organisms. Such effects especially well documented in marine animals, included strangulation and other physical malformations caused by large plastic materials found in the environment,

gastrointestinal tract full of plastics instead of food and death by starvation, among others (Gregory, 2009; Wright *et al.,* 2013). Such evidences caused a high concern among the scientific community and latter among the general public leading to an increase of research and other effects to find solutions for this major problem.

The formation of small particles reaching the micro or lower scale, resulting from the fragmentation of larger debris is a slow process which causes the formation of so-called secondary microplastics, distinguishing them from primary microplastics, whose characteristic is to intentionally be produced to have a microscopic size (Cole *et al.,* 2011). The United States of America (U.S.A.) National Oceanic and Atmospheric Administration (NOAA) defined microplastics as particles whose size is smaller than 5 mm.

Microplastics have been widely found, including in remote regions, such as Arctic, were anthropogenic activity is poor (Ivar do Sul & Cost, 2014). However, regions with a great human presence show higher concentrations. In fact, Martins & Sobral (2011) found concentrations up to 197 particles (size < 5 mm) per m^3 in Portuguese beaches sediments. Besides, concentrations up to 1 particle per 25 cm^3 were found in deep sea sediments of the Atlantic Ocean (Van Cauwenberghe *et al.,* 2013). Concentrations up to 9800 particles per m^3 and 779 particles per liter were found in water samples from the northeastern Pacific Ocean (Desforges *et al.,* 2014) and in Norwegian coastal waters, respectively (Norén & Naustvoll, 2010).

Due to their characteristics, microplastics are ingested by several animals, such as fish (Gregory, 2009), planktonic organisms, larval fish and copepods (Hollman *et al.,* 2013), birds (Van Franeker *et al.,* 2011), among others. Colour, size, density, are among the factors capable of affecting the bioavailability of microplastics in the marine environment (Wright *et al.,* 2013). These factors allow many organisms to be able to ingest such debris (Fendall & Sewell, 2009). Plastics may then integrate the marine food chain, potentially leading to effects observed in a wide range of organisms (Derraik, 2002). Gregory (2009) revealed that most of the injuries caused by plastic debris were internal and external abrasions, ulcers, digestive tract blockages leading to satiation, starvation and physical deterioration, reduction of reproductive fitness, drowning, reduced predator avoidance, reduced feeding capacity, and in extreme cases, the death of a large number of organisms. Besides, chemical toxic effects resulting from the ingestion of microplastics can also be observed. In fact, Oliveira *et al.,* (2013), revealed that microplastics inhibit AChE activity in a study using the common goby *Pomatoschistus microps*. They also observed toxic effects when microplastics were combined with pyrene, concluding that the study of the toxic effects of microplastics either alone or combined with many environmental contaminants is of particular interest. Additionally, plastics may contain chemicals used in their production or introduced intentionally and may incorporate

pollutants from the ecosystem, potentially altering their toxicity (Andrady, 2011). Several studies demonstrate that there is an interaction with persistent organic pollutants (POPs) (Engler, 2012), polycyclic aromatic hydrocarbons (PAHs) (Teuten *et al.,* 2007; Oliveira *et al.,* 2013) and some other environmental contaminants which may increase or decrease microplastics toxicity. Despite the studies that have been carried out on the effects of microplastics on marine organisms and ecosystems, a considerable amount of works still needs to be done. For example, there is a lack of knowledge on the socio-economic impacts of microplastics, especially concerning the fishery industry, effects at population level, influence of hydrodynamic factors on the transport and accumulation of debris and on the interaction between microplastics and other environmental pollutants (Galgani *et al.,* 2013).

1.4. Assessment of the biological effects of environmental contaminants on marine animals

The biological effects of environmental contaminants on the marine biota may be assessed both in the field and/or in the laboratory, often in a complementary way. Field studies include monitoring of wild populations to assess their health status, studies of communities, interspecific relationships, among several other types of studies. In the laboratory, the biological effects of environmental contaminants may be evaluated by different approaches, including toxicity bioassays with model and/or wild organisms using approaches and parameters at distinct levels of biological organization. Among these, fish bioassays are among the most used ones. In these bioassays, the effects on mortality, growth, reproduction, behaviour, health condition, physiological functions, and molecular alterations may be under investigation. In the last decades, the use of behavioural parameters and biomarkers has been also widely used.

In Ecotoxicology, the term “biomarker” has been used to indicate a biological parameter measured at individual and/or sub-individual levels in an organism or in its sub-products (*e.g.* blood, urine, feathers) able to provide an indication of its health and/or performance (Van Gestel & Brummelen, 1996; Vieira *et al.,* 2009). In the present Thesis, it will be used too, including behavioural parameters. Biomarkers are very important because they allow the early detection of adverse effects, allowing the adoption of preventive measures before the effects becoming severe and difficult to overcome (Van der Oost *et al.,* 2003). Some of them also allow the investigation of the mechanisms of toxicity contributing to the effects observed at individual or higher levels of biological organization, among other. Thus, they are suitable effect criteria in toxicity bioassays.

The activity of the enzymes cholinesterases (ChE), glutathione S-transferases (GST) and ethoxyresorufin-O-deethylase (EROD), and peroxidation levels (LPO) are among the most used biomarkers in bioassays with fish and other aquatic organisms. ChE are a family of enzymes with high affinity for choline esters (Romani *et al.,* 2003). In vertebrates, ChE include acetylcholinesterase (AChE), the enzyme that makes the degradation of the neurotransmissor acetylcholine in cholinergic synapses, being also present in the blood and several other tissues, and pseudocholinesterase, also known as butyrylcholinesterase, that is a less specialized enzyme that is part of the defenses against chemical and other stress (Klaassen, 2008). In Ecotoxicology, ChE were first used as specific biomarker for organophosphate and carbamate compounds because they are strongly inhibited by low concentrations of these agents, and are still used for this purpose (Rickwood & Galloway, 2004; Oliveira, C *et al.,* 2013). However, because several other contaminants are also able to inhibit these enzymes at concentrations that may be found in the environment and several evidences showed that this happens in real scenarios, a more wide use for these enzymes was purposed in the 1990s (Payne *et al.,* 1996; Labrot *et al.,* 1997; Guilhermino *et al.,* 1998) and used since then (*e.g.* Romani *et al.,* 2003; Vieira *et al.,* 2009; Oliveira *et al.,* 2013). In the most part of the situations, the inhibition of ChE activity is used as indicative of neurotoxicity.

For the majority of xenobiotic compounds, the phase I reactions are catalyzed by microsomal monooxygenase (MO) enzymes, which contains cytochrome P450. These enzymes facilitate the excretion of certain compounds by phase I metabolism, as it transforms lipophilic xenobiotics to more water-soluble compounds (Vehniäinen *et al.,* 2012). Production of the protein P4501A resulting from cytochrome CYP1A gene activation can be assessed by measuring its enzymatic activity. The activity of the 7-ethoxyresorufin O-deethylation (EROD) enzyme is considered the most sensitive catalytic probe for determining the inductive response of the cytochrome P450 system in fish (Van der Oost *et al.,* 2003; Vehniäinen *et al.,* 2012). The induction of CYP1A catalytic activities may be used both for the assessment of exposure and as early-warning sign for potentially harmful effects of many pollutants (Van der Oost *et al.,* 2003; Klaassen, 2008; Vieira *et al.,* 2009).

The glutathione-S-transferase (GST) is part of biotransformation reactions of phase II promoting a great increase of hydrosolubility of several xenobiotics and thus promotes the excretion of external chemicals (Van der Oost *et al.,* 2003). GST acts in this process by catalyzing the conjugation of those compounds with GSH (tri-peptide glutathione, ɣ-gluthamyl-cysteinyl-glycine) (Van der Oost *et al.,* 2003). GST activity has been widely used as a biomarker of fish exposure to toxic compounds (Vieira *et al.,* 2008; Vieira *et al.,* 2009).

Lipid Peroxidation (LPO), the oxidation of polyunsaturated fatty acids and cholesterol, is an important consequence of oxidative stress (Van der Oost *et al.,* 2003). Oxidative stress has been observed after exposure to several environmental contaminants (or their metabolites) (Van der Oost *et al.,* 2003). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defenses (Costa & Moradas-Ferreira, 2001). Since many environmental contaminants exert toxic effects related to oxidative damage, LPO activity determination has constituted an important biomarker in ecotoxicity tests (Vieira *et al.,* 2009; Oliveira *et al.,* 2012).

Behaviour can be defined as a conjunct of actions made by organisms and what surrounds them, under certain circumstances to assess environmental quality (Hellou, 2011). Besides common toxicological effects observed in organisms (*e.g.* survival, growth), behavioural endpoints such as reproduction (Martins *et al.,* 2013), feeding (Moreira *et al.,* 2006), avoidance (De Lange *et al.,* 2006; Oliveira *et al.,* 2013), swimming (Gravato & Guilhermino, 2009; Oliveira *et al.,* 2013), among others, have been gaining recognition in scientific community because of their great sensitivity (Hellou, 2011). Behavioural tests are a cost-effective procedure, simple to perform and revealed to have a great ecological relevance (Hellou, 2011).

1.5. Objectives and structure of the dissertation

The central objective of the present study was to investigate the influence of microplastics on the short-term toxicity of cephalexin to juveniles (0^+ age group) of the common goby (*P. microps*) at two temperatures (20 and 25ºC). The following hypotheses were tested: (i) water concentrations of cephalexin in the low ppm range are toxic to *P. microps* juveniles; (ii) the presence of microplastics in the water influence the toxicity of cephalexin to the test organism; (iii) the 5ºC raise of temperature in the range tested (20ºC – 25ºC) modulates the effects of the tested substances.

The common goby (*P. microps*) was selected as model organism for this study mainly because it has a wide geographical distribution going from the Atlantic coast from Norway to Morocco, in the North Sea, in the Baltic Sea, and in the western part of the Mediterranean Sea (Pockberger *et al.,* 2014), has an high abundance and high fecundity (Quintaneiro *et al.,* 2008). The species is predominantly found in coastal ecosystems (Quintaneiro *et al.,* 2008). In the early phases of its life cycle, *P. microps* is mainly planktivorous, predominantly feeding on copepods and other zooplankton species (Pockberger *et al.,* 2014). Because of its small size, the common goby is an important

prey for larger predators during its entire life cycle. As a result of its middle trophic position within the food chain, the population size of *P. microps* can influence the population size of species in lower and higher trophic levels (Leitão *et al.,* 2006; Quintaneiro *et al.,* 2008), making it a keystone species (Pockberger *et al.,* 2014). For all these reasons, *P.microps* has been used as test organism in fish bioassays and as bioindicator in monitoring studies of the effects induced by the exposure to environmental contamination (Monteiro *et al.,* 2007; Quintaneiro *et al.,* 2008; Vieira *et al.,* 2009; Oliveira *et al.,* 2013).

Cephalexin was selected for this study due to its large global consumption allowing its frequent appearance in aquatic environments (Li, 2013). Fluorescent polyethylene plastic microspheres (MP) were selected as model for microplastics because polyethylene is one of the plastic types more produced, used and found in the marine environment (Andrady, 2011; Plastics Europe, 2013), their fluorescence may allow the determination of their concentrations in test media in a cost-effective way, and they were successfully used in previous works of our research group (Oliveira *et al.,* 2013).

The Thesis is divided in six chapters: Chapter I – Introduction; Chapter II – Material and Methods; Chapter III – Results and discussion; Chapter VI – Main Conclusions; Chapter V – List of References and Chapter VI - Annex. The first chapter introduces the problem of environmental contamination by emerging contaminants, particularly antibiotics and microplastics, some of the approaches used to assess the effects of environmental contaminants on the biota, and present the objectives, hypotheses to be tested and the structure of the dissertation. In the second chapter, the methods and procedures used in the experimental work are described. In the third chapter, the results obtained are presented and discussed. In the fourth chapter, the main conclusions are summarized. In the fifth chapter, the list of references corresponding to the citations made in the text is indicated and finally, the sixth chapter corresponds to the annex.

CHAPTER II

2. Material and methods

The experimental work was divided in four main phases: (i) get training and validate the methodologies for further use during the experimental work; (ii) optimization of the spectrophotometry and spectrofluorometry methods to determine the actual concentrations of cephalexin and MP, respectively in artificial sea water (ASW); (iii) preliminary assessment of cephalexin concentrations inducing toxic effects on *P. microps* juveniles; and (iv) influence of cephalexin alone and in the presence of MP to *P. microps* assessed at 20ºC and 25ºC.

2.1. Test substances and other chemicals

The test substances used in the present study were cephalexin hydrate (CAS no. 15686-71-2), purchased from Sigma-Aldrich Chemical (Germany) and used with a degree of purity of 100% and red fluorescent polyethylene microspheres with a size range between 1 and 5 µm purchased from Cospheric (USA).

The ASW was prepared with sea salt Tropic Marin® Sea Salt from Germany. The chemicals for enzymatic analysis were of analytical grades, acquired from Sigma–Aldrich Chemical (Germany), Bio-Rad (Germany), or Merck (Germany).

2.2. Collection of *P.microps* juveniles and acclimatization to laboratory conditions

Juveniles (0+ age group) were collected in the Minho River estuary (NW of Portugal - 41°52'43.74"N; 8°49'51.91"W) in November 2013, February 2014, May 2014 and July 2014. They were collected in this estuary because it is considered a relatively low impacted estuary and was found to be an adequate source of juveniles of this species to be used in toxicity bioassays (Ferreira *et al.,* 2003; Vieira *et al.,* 2008; Vieira *et al.,* 2009; Oliveira *et al.,* 2013). The sampling was performed at low tide using a hand operated net. Fish were transported to the laboratory within the lowest time possible in thermally isolated boxes with local water and aeration (pump Nirox, x-19, Malaysia).

At the time of the capture, the following parameters were measured using a field probe device (HACH® HQ40d, USA) (Figure 2): temperature, pH, dissolved oxygen (D.O) and salinity (Table A1).

Figure 2: Capture of *Pomatoschistus microps* juveniles in the Minho River estuary and probe used to measure some abiotic parameters

Once in the laboratory, fish were acclimatized to laboratorial conditions for at least one week before being used in the bioassays. They were maintained in a room with a photoperiod of 16 h light (L): 8 h dark (D) (light: 4.25 E/m^2s, provided by a solar-like spectrum light) and room temperature between a minimum of 17.1ºC and a maximum of 22.3ºC was measured with the aid of a DATA LOGGER (PCE-HT 71N Humidity/Temperature) supplied by the PCE Instruments (United Kingdom). The fish used in the definitive bioassays were acclimatized after this first period to the temperature (at 20ºC or 25ºC according their further use) and other conditions of the bioassays in temperature and photoperiod controlled chambers (Bronson PGC 1400, The Netherlands), at 16 h L: 8 h D (6.9 E/m^2s of total photon flux provided by solar-like spectrum light). Fish were maintained in aquaria (about 200 per aquarium) filled with approximately 68 liter of ASW (Figure 3) prepared by dissolving aquarium salt (Tropic Marin® Sea Salt, Germany) in distilled water until reaching 18 practical salinity units (PSU), corresponding to 18 g/l (measured with seawater refractometer HI 96822 from Hanna Instruments, USA). The ASW was continuously filtered (Eheim classic, Germany), partially renewed twice a week, and air was continuously supplied (pump TAGUS 2000, Portugal). Fish were fed daily *ad libitum* with commercial fish food (Aquapex Tropicmix, Orni-ex, Portugal) and abiotic parameters were measured every 24 h during the acclimatization period (Table A2).

Figure 3: Aquaria used to maintain fish along the acclimatization period.

2.3. Optimization of the conditions to determine the actual concentrations of cephalexin and its decay in test media

The spectrophotometry method Mohammad (2009) was tested for its potential use in ASW in order to determine the concentrations of cephalexin and its decay during the exposure period. Three solutions of cephalexin (10 mg/l) were prepared in ultra-pure (u.p.) water and three in ASW. Each one was serial diluted to obtain six additional cephalexin concentrations (10, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 mg/l). The UV-Vis absorption spectra of each solution were performed in a JASCO® V-630 UV/Vis (USA) spectrophotometer. The Pearson correlation coefficient (r) was used to measure the correlation between the two variables (cephalexin concentrations and absorbance at 260 nm values). This wavelength was used because cephalexin has an absorbance peak at 260 nm (Florey, 1975). The absorbance values were plotted against the corresponding cephalexin concentrations. Hereafter, a linear regression model was fitted to the data using the cephalexin concentration as dependent variable and the absorbance as independent variable to obtain the absorbance *versus* concentration calibration curve. The concentrations of cephalexin in the samples of test medium collected at the beginning of the bioassays were determined from the equation of the linear model adjusted to the calibration curve. The decay of the test substance was determined directly from the absorbance values.

2.4. Determination of microplastics concentrations and decay

According to the manufacturer, the MP used have an excitation wavelength of 470 nm and an emission wavelength of 588 nm. Thus, these were the wavelengths used to

determine the concentrations of MP in test media and their decay along the bioassay. All the determinations were performed in a JASCO® FP-6200 spectrofluorometer (USA). Three independent suspensions of MP in ASW with a concentration of 12 mg/l were prepared. Each one was serial diluted 1:2 (v/v) in ASW to obtain additional suspensions with concentrations between 12 mg/l and 0.012 mg/l. However, only the suspensions with concentrations between 1.5 mg/l and 0.012 mg/l were selected for the calibration curve aimed at further determining the MP concentrations in test media during the bioassay. The selection of the concentrations to be used was done in order to have the nominal concentration of MP to be used on the bioassays (0.184 mg/l) in the middle of the curve to allow a more accurate calculation of the actual concentrations on the test media. The readings of the fluorescence of blank (ASW only) and of the MP suspensions were performed using the excitation and emission wavelength previously described. After subtracting the blank values, the Pearson correlation coefficient (r) was used to measure the correlation between the two variables, and a linear regression model was fitted to the data using the MP concentrations as dependent variable and the fluorescence values as independent variable. The potential decay of solutions containing MP in test media along the bioassays was assessed directly from the fluorescence readings at 470/588 nm (excitation and emission wavelength, respectively), after subtracting the fluorescence of blanks (ASW), while the MP actual concentrations were determined from the calibration curve from the fluorescence values.

2.5. Optimization of the conditions for use in the bioassays

A first bioassay was carried out to get training in the bioassays methodology to optimize the experimental conditions, and to find the lowest range of cephalexin concentrations inducing toxic effects on *P. microps* juveniles after 96 h of exposure. The juveniles used in this bioassay were collected in November 2013. The bioassay followed in general the OECD guideline nº 203 (OECD, 1992), except for the number of organisms used per treatment (only 6 fish per treatment were used for ethical reasons). Due to the high susceptibility to manipulation of *P. microps* juveniles, they were measured from the top of the head to the begin of the caudal fin and weighed using an analytical balance (Kern ABS-N, KERN & SOHN GmbH, Germany) at the end of the bioassay only. The bioassay was carried out under a 16 h L: 8 h D (4.25 E/m^2s, light provided by a solar-like spectrum light), with a room temperature variation between 17 and 22.2ºC. A stock solution of cephalexin hydrate was prepared in ASW with a concentration of 5 mg/l. The limit of solubility of cephalexin hydrate in water is 13.5 mg/ml (Florey, 1975) and thus no

solvent was used. Cephalexin nominal concentrations (5, 2.5, 1.25, 0.625 0.313 and 0.156 mg/l) were prepared by serial dilution of the stock solution in ASW prepared as indicated above (section 2.2.) with a salinity of 18 PSU. The control treatment was ASW only. Fish were randomly distributed by the test treatments, and were individually exposed for 96 h in 1 liter glass beakers filled with 500 ml of each test solution. All the beakers were sealed to avoid evaporation and substances losses, with the exception of the air tube entrance (Figure 4). The glass beakers were previously washed with HNO_3 10% and then with distilled water. Air supply was continuously provided from an air compressor (Nitto Kohki® médo - Japan) and distributed individually to the test beakers. The test medium was not renewed and no food was provided to the fish during the exposure period. The effect criteria were mortality, post-exposure predatory performance, hereafter indicated as "predatory performance" (section 2.5.1), and four sub-individual biomarkers (section 2.5.2), namely the activity of enzyme AChE as indicative of neurotoxicity; the activity of the enzymes EROD and GST as indicative of biotransformation changes (phase I and II, respectively) and LPO levels as indicative of oxidative damage. Fish were considered dead when there was no visible movement and if a gentle touch with a plastic micropipette produced no reaction (OECD, 1992).

Water temperature, pH and dissolved oxygen measured using a probe device (HACH® HQ40d, USA) and fish mortality were monitored at 0, 24, 48, 72 and 96 h and fish were regularly observed along the day. Samples of each beaker were collected at the beginning of the bioassay and at each 24 h until the end of the bioassay to determine the concentration of the test substance.

2.5.1 Post-exposure predatory performance

After 96 h of exposure to cephalexin, the predatory performance of each fish was assessed through a post-exposure predatory test previously developed and validated in the scope of the project SIGNAL. Previously, *Artemia franciscana* cysts were hatched in ASW with salinity of 37 PSU with aeration. Nauplii used in the predatory performance assay were about 24 h old. After completing 96 h of exposure, each fish was removed from the test beaker and put in a predator-prey chamber (14 cm diameter, 11.5 cm high) containing 300 ml of clean ASW. After five minutes of fish adaptation to the new conditions, 12 preys (*A. franciscana* nauplii) were added to the chamber (from the results obtained in the first two bioassays, it was decided to use 30 preys in further bioassays), corresponding to the initial number of preys. After three minutes, the fish was removed

and the number of nauplii left in the container was counted. The percentage of ingested prey by each fish was then calculated as: ((initial number of prey – prey remaining in the chamber after fish removal) / initial number of prey) x 100. Each fish was put back in its test beaker and left resting for two hours.

2.5.2. Biomarkers

All living fish were sacrificed by decapitation under cold induced anaesthesia. To avoid possible influence with biomarker determinations, no chemical anaesthetics were used. From each fish, the head and the body were isolated on ice and homogenized separately using an Ystral® D-79282 (Germany) homogenizer in appropriate ice cold buffers (0.1M; pH = 7.2 and 7.4 for the head and body respectively). The amount of buffers used depended on the size of the fish, varying between 0.5 and 1 ml for head samples and between 0.7 and 3 ml for body samples. All the centrifugations were done using an Eppendorf 5810R centrifuge (Germany). The homogenate of the head was centrifuged for 3 minutes, at 3300 g (4ºC). The supernatant was carefully collected and stored at -80ºC for further determination of AChE activity. The homogenate of the body was divided in three aliquots: one for LPO determination that was immediately stored at -80ºC without centrifugation to preserve the organelles; and the others were separately centrifuged for 20 minutes at 10000 g (4ºC) to obtain the post-mitochondrial fraction for GST and EROD determination. The supernatants were carefully collected and stored at -80ºC for further analysis.

The protein content of the samples was assessed by the Bradford method (Bradford, 1976) adapted to microplate (Frasco & Guilhermino, 2002) in BIO-TEK® microplate reader; model POWERWAVE 340 (USA) and standardized to 0.5 mg protein/ml (Oliveira *et al.,* 2013). This procedure was not made for LPO and EROD samples since all the protein was necessary to carry out the analysis (small fish body). The method consists in the addition of an acidic dye to protein solution, and subsequent measurement of the absorbance at about 595 nm. A differential colour change of a dye occurs in response to various concentrations of protein. The standard curve was done using γ-globulin bovine as protein standard. Ninety-six well microplate (Costar®, USA, 0.320 ml of capacity) was used. To obtain the protein standard curve, a standard solution of γ-globulin bovine (1 mg/ml) was prepared in advance. From it, 0, 0.002, 0.005 and 0.01 ml were put in a series of microplate wells, with an additional well empty (negative control); then, 0.010, 0.008, 0.005 and 0 ml of u.p. water were added to each well (following the same order). From each biological sample, 0.01 ml was introduced in

different well series. Then, 0.250 ml of a diluted Bio-Rad solution (1 Bio-Rad reagent: 4 u.p. water (v/v)). All the determinations were made in triplicate. After 15 minutes of mixing, the optical density (O.D.) was read at 595 nm in a BIO-TEK® powerwave 340 (USA) microplate reader. After the determination of the biomarker analysis, and because the protein standardization procedure was made by dilution after the spectrophotometric reading, the concentration of protein in the samples was determined again to express enzymatic activities and LPO levels per mg of protein in a more accurate way.

AChE activity determination was performed in the supernatant of fish head homogenates previously prepared as described above. In the experimental conditions used in the present study, *P. microps* head samples contain mainly AChE as indicated in previous studies from our team (Monteiro *et al.,* 2005). AChE activity was determined according to the method of Ellman (Ellman *et al.,* 1961) adapted to microplate (Guilhermino *et al.,* 1996). The Ellman´s method is based on the formation of a yellow color whose O.D. raises with the increase of acetylthiocholine degradation caused by the action of AChE present in the samples. Acetylcholine degradation forms acetyl and thiocholine. In the presence of the cromophore 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), the thiocholine reacts with it, producing 5-thio-2-nitro-benzoic acid that has a yellow color that can be measured by spectrophotometry at 412 nm. For AChE activity determination, a solution (0.075 M) of acetylthiocholine in u.p. water was prepared in advance in dark conditions and maintained at 4ºC (also in dark conditions) for a maximal period of two weeks (solution A). A solution of DTNB (0.01 M) (with 0.017 M of sodium bicarbonate) in phosphate buffer 0.1 M, pH = 7.2 was also prepared in the same conditions and maintained at 4ºC for a maximal period of two weeks (solution B). In the day of the assay and before enzymatic determinations, a reaction solution was prepared by adding 0.2 ml of the solution A and 1 ml of the solution B to 30 ml of phosphate buffer (0.1 M, pH = 7.2). The enzymatic reaction starts with the addition of 0.05 ml of head homogenate supernatant previously defreeze and gently mixed, into a well of a ninety-six wells microplate (Costar®, USA, 0.320 ml of capacity) and 0.250 ml of the reaction solution. A first well column was left empty to calibrate the microplate reader (BIO-TEK® powerwave 340) and a second well column contained only 0.05 ml of K-phosphate buffer (0.1 M, pH = 7.2) to be used as calibrate for the degradation of acetylthiocholine without the enzyme action. All the determinations were made in triplicate. The kinetic reaction was measured for 5 minutes at 412 nm. The enzymatic activity was expressed as nanomoles of thiocholine formed per minute per mg of protein (nmol/min/mg protein).

EROD activity was determined by the method of Burke & Mayer (1974). A solution of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) in phosphate buffer 0.1 M, pH = 7.4 (solution C), and a reaction buffer containing 1.5 ml of a ethoxyresorufin stock

solution (410.85 mM) in 49.5 ml of phosphate buffer (0.1 M, pH = 7.4) were prepared. Then, 0.1 ml of each biological sample, 0.01 ml of the solution C and 1 ml of the reaction buffer were added to a glass cuvette. For the blank, 0.1 ml of phosphate buffer (0.1 M pH = 7.4) instead of the biological sample was used. The kinetic was determined in a JASCO® FP-6200 spectrofluorometer (USA) for 6 minutes; the wavelengths of excitation and emission were 530 nm and 585 nm, respectively. The enzymatic activity was expressed as pmol of formed resorufin per minute per mg of protein (pmol/min/mg protein).

GST activity determination was performed according to Habig *et al.* (1974) adapted to microplate (Frasco & Guilhermino, 2002). A solution (0.060 M) of 1-chloro-2, 4-dinitrobenzene (CDNB) in ethanol was prepared in advance and maintained on ice (solution D). A solution of glutathione (0.010 M) in phosphate buffer (0.1 M, pH = 6.5) was also prepared and maintained in the same conditions (solution E). A reaction solution was prepared by adding 1.5 ml of the solution D and 9 ml of the solution E to 49.5 ml of phosphate buffer (0.1 M, pH = 6.5). After setting the protein of each sample to 0.5 mg protein/ml using the Bradford method, 0.05 ml of the body homogenate supernatant, previously defreeze and mixed, were put in a well ninety-six wells microplate (Costar®, USA, 0.320 ml of capacity) and 0.250 ml of the reaction solution were add. A first well column was left empty to calibrate the microplate reader (BIO-TEK® powerwave 340) and a second well column contained only 0.05 ml of K-phosphate buffer (0.1 M, pH = 6.5) to be used as blank. GST activity was determined by the conjugation of the thiol group of the GSH catalyzed by GST with CDNB leading to an increase of the absorbance value at 340 nm. The kinetic reaction was recorded at 340 nm for 5 minutes on a BIO-TEK® powerwave 340 microplate reader (USA). All the determinations were made in triplicate. GST activity was expressed as nmol of conjugate formed per minute per mg of protein (nmol/min/mg protein).

LPO levels were measured by quantification of thiobarbituric acid reactive substances (TBARS) (Ohkawa *et al.,* 1979). For LPO activity determination, a solution of thiobarbituric acid (TBA) 0.73% (solution F) was prepared by adding 0.73 g of TBA to 100 ml of u.p. water. The solution was maintained in dark conditions after its preparation. A solution of Tris-HCl (0.060 M, pH = 7.4) with diethylenetriaminepentaacetic acid (DTPA) (0.0001 M) whose pH was stabilized at 7.4 using sodium hydroxide (NaOH) (solution G), and a solution of trichloroacetic acid (TCA) 12% in u.p. water (solution H) were also prepared and maintained on ice. The experience started with the addition of 0.200 ml of the body sample, 1 ml of solution F, 0.800 ml of solution G and 1 ml of solution H in a 15 ml falcon tube (tubes with solution F, G and H and 0.200 ml of phosphate buffer 0.1 M, pH = 7.4 as blank). After incubating all the tubes previously prepared in a water bath at a

temperature of 100ºC for 60 minutes, 2 ml of each recently prepared sample were transferred to a microtube and centrifuged for 5 minutes at 14000 g (25ºC). The absorbance was read at 535 nm, using a JASCO® V-630 UV/Vis (USA) spectrophotometer. Then, the protein levels of each sample were determined and LPO levels were expressed as nmol of TBARS formed per mg of protein (nmol TBARS/mg protein).

2.6. Preliminary cephalexin bioassay

The fish used in this bioassay were captured in February 2014. This bioassay was done as a preliminary assessment of cephalexin concentrations inducing toxic effects on *P. microps* juveniles. Nine fish were used per treatment. The bioassay was carried out under a photoperiod of 16 h L: 8 h D and water temperature of 21.0 ± 0.34ºC. The nominal concentrations of cephalexin tested were 0.313, 0.625, 1.25, 2.5 and 5 mg/l. They were prepared by serial dilution of a stock solution prepared in ASW (18 mg/l). All the other conditions were similar to those described in section 2.3.

2.7. Influence of microplastics and temperature on the cephalexin toxicity

The fish used in these bioassays were captured in the Spring May and July 2014. Two bioassays (one at 20ºC and the other at 25ºC) were carried out to investigate the effects of cephalexin, the influence of MP on its toxicity, and the influence of temperature in the chemicals toxicity. All the bioassays were carried out in photoperiod of 16 h L: 8 h D and temperature (20ºC or 25ºC) controlled chambers (Bronson PGC 1400, The Netherlands) with fish previously acclimatized to the test temperature for one week (after the initial acclimatization period), and the test media was ASW. The bioassays consisted in the following treatments: control (ASW only), four cephalexin concentrations (1.25, 2.5, 5, 10 mg/l), MP alone (0.184 mg/l) and the same four concentrations of cephalexin (1.25, 2.5, 5, 10 mg/l) combined with 0.184 mg/l of MP. Cephalexin test concentrations were prepared as described in section 2.5 and 0.184 mg/l was added to all the test beakers except for the control group and the beakers of four cephalexin concentrations alone. This MP concentration was selected based on the results of a previous study with *P. microps* early juveniles (Oliveira *et al.,* 2013). Twelve fish randomly selected were assigned to each treatment. All the other conditions and procedures were similar to those described in section 2.5.

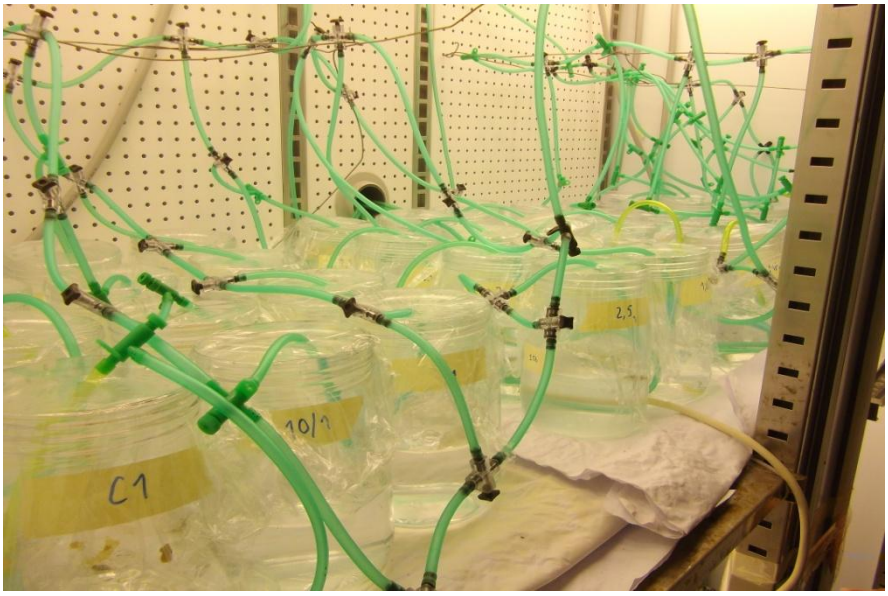


Figure 4: Glass recipient properly sealed and aeration system used in the 96 h of exposure to cephalexin in the absence and presence of MP in the photoperiod and temperature controlled chamber.

2.8. Statistical analysis of data

The percentages of predatory performance were transformed with an arc sin transformation (Zar, 1996). All the other variables were checked with a Kolmogorov-Smirnov test to investigate the normality of variable distribution and a Levene's test was used to investigate the homogeneity of variances. If these assumptions of the Analysis of Variance (ANOVA) were not checked, data transformations to correct departures were used. For each parameter of the four bioassays, different treatments were compared by one-way ANOVA (1-ANOVA), followed by the Tukey test when significant differences were found. At 20ºC and 25ºC, a two-way ANOVA (2-ANOVA) was carried out to investigate differences among treatments, presence of MP and the potential interaction between them. A post-hoc Tukey test was performed when significant differences between treatments were detected by the 2-ANOVA. Finally, in order to understand if temperature changes affect the toxicity of the tested substance, a 2-ANOVA was carried out to investigate differences among treatments, temperatures and the potential interaction between them. The Student t-test was performed to see if statistical significant differences between fish exposed to the two distinct temperatures were observed. Statistical analyses were performed using the SPSS 20.0 software package. The significance level was 0.05.

CHAPTER III

3. Results and Discussion

3.1. Spectrophotometry method to assess cephalexin concentrations

The UV-vis partial spectra of representative cephalexin solutions prepared in u.p. water and in ASW are shown in Figure 5.

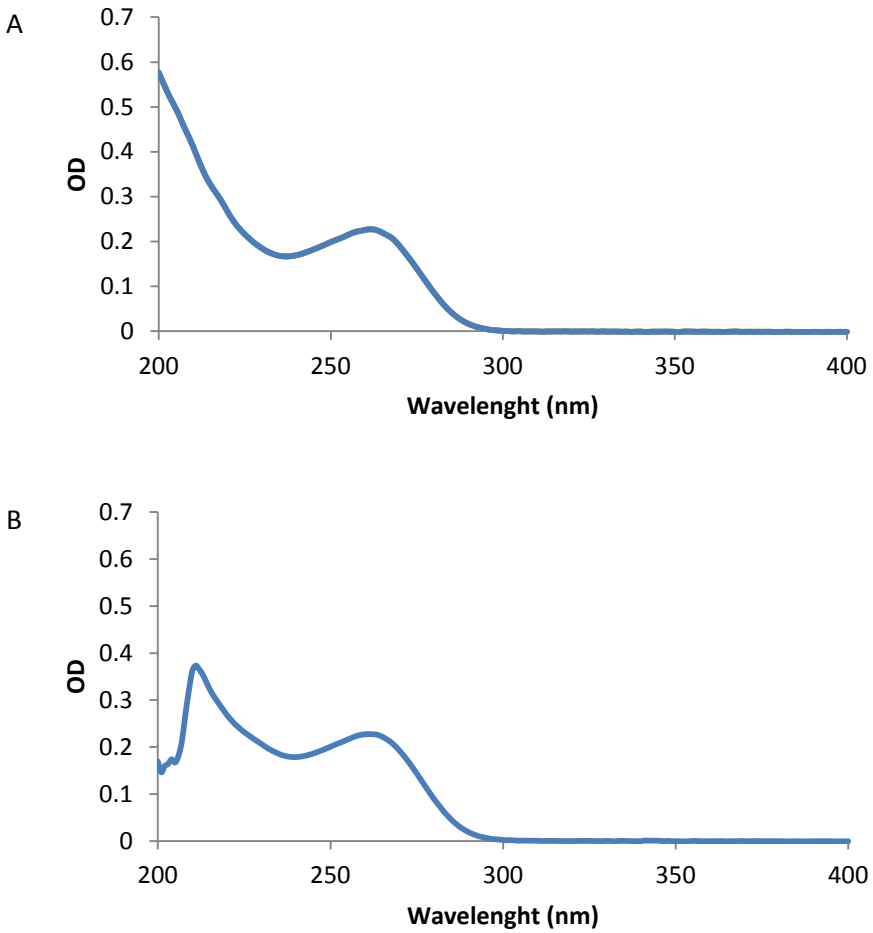


Figure 5: UV-vis absorption spectra of two cephalexin solutions, one in ultra-pure water (A) and the other in artificial sea water (B), with a concentration of 10 mg/l in both cases. OD – optical density units

The typical spectrum of cephalexin has a peak of absorbance at 260 nm (Florey, 1975). In both spectra of Figure 5 corresponding peaks are visible. Furthermore, the absorbance at 260 is 0.225 in u.p. water and 0.227 in ASW. Therefore, the u.p. and ASW spectra are very similar. A positive and significant correlation between the nominal cephalexin concentrations and their absorbance was found in both u.p. water (N = 21, r =

0.974, $p < 0.05$) and ASW ($N = 21$, $r = 0.995$, $p < 0.05$). The linear model adjusted to the calibration curve in ASW was: cephalexin concentration (mg/l) = - 0.015 + 46.521 x absorbance (OD units) (Figure 6). The linear model fitted to the corresponding curve made in u.p. water was comparable: cephalexin concentration = - 0.376 + 47.058 x absorbance (OD units) (Figure 7). Overall, these findings indicate that this spectrophotometry method (Mohammad, 2009) can be used to measure the concentrations of cephalexin in ASW in the range of concentrations used for the calibration curves.

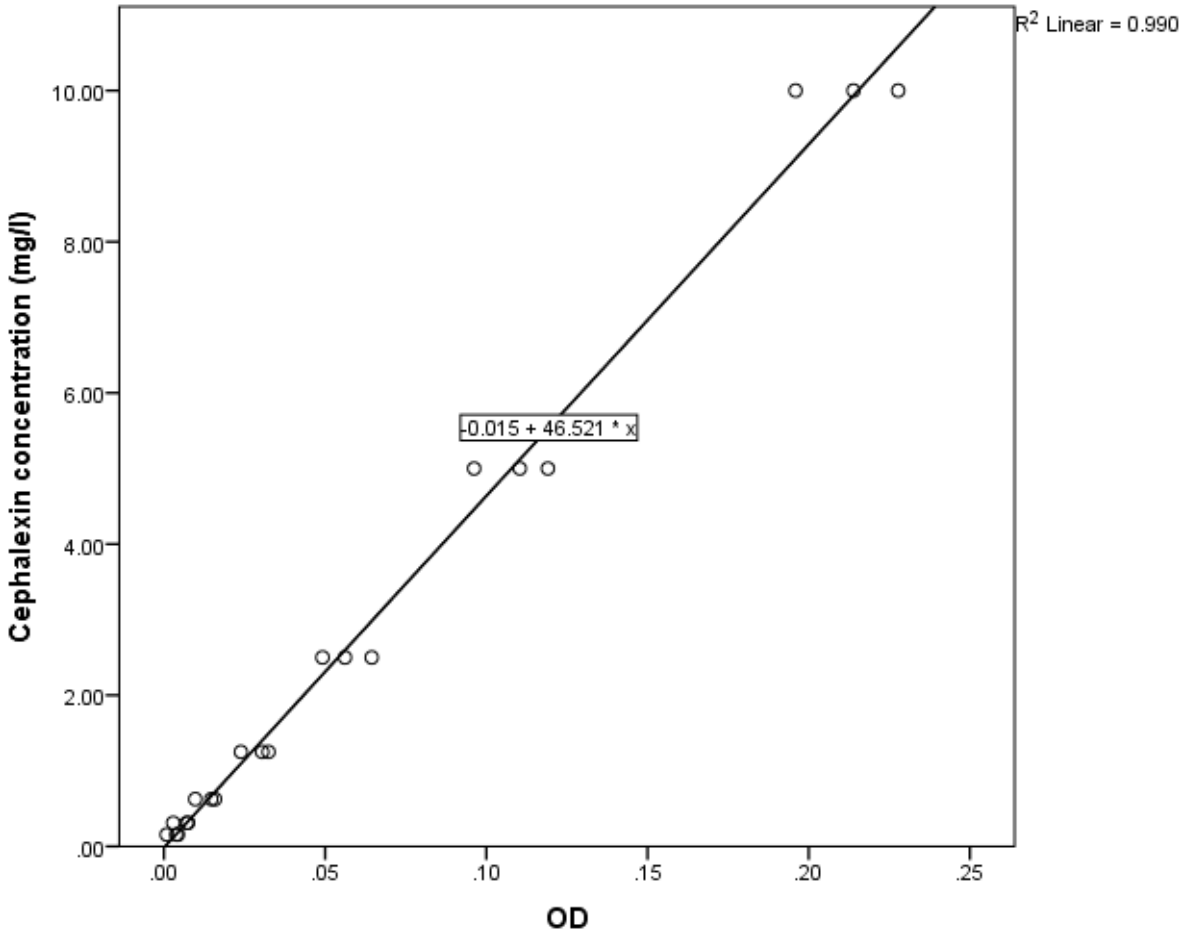


Figure 6: Linear model fitted to the calibration curve (260 nm absorbance values *versus* nominal concentrations of cephalexin) in artificial sea water. OD – absorbance measured in optical density units. R^2– coefficient of determination.

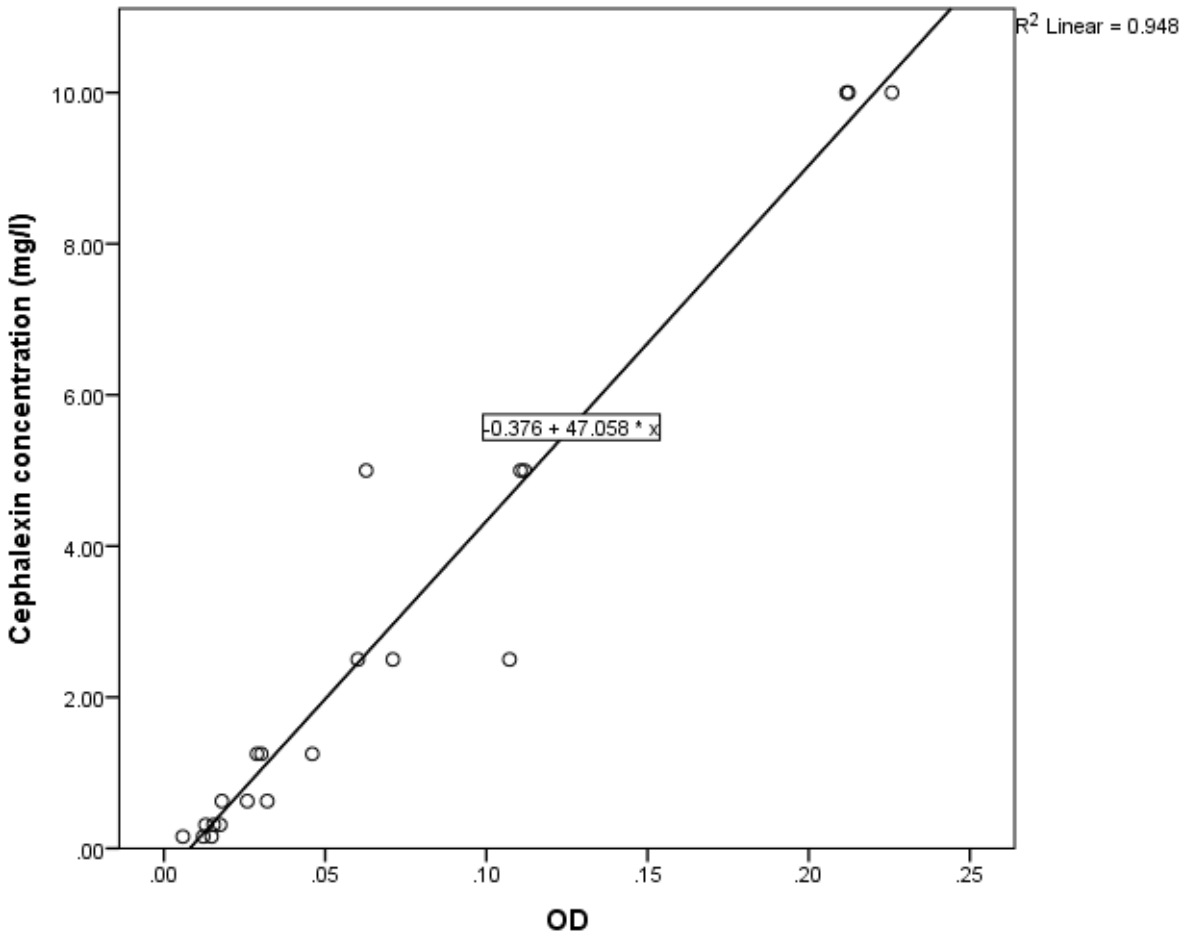
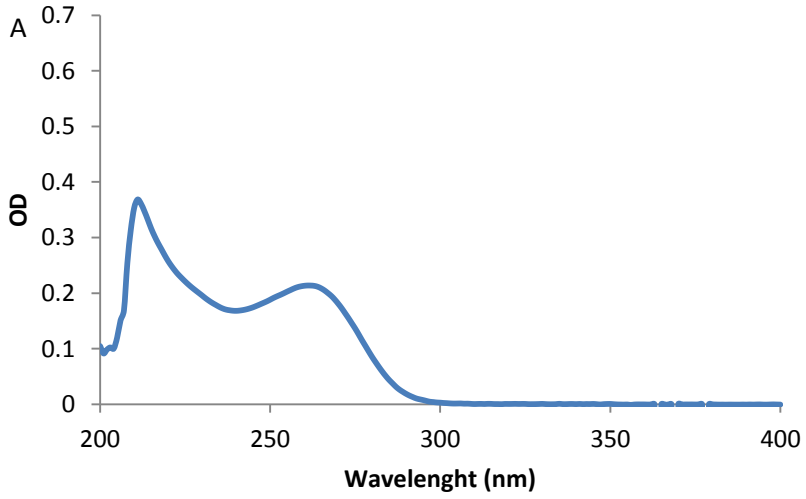


Figure 7: Linear model fitted to the calibration curve (260 nm absorbance values *versus* nominal concentrations of cephalexin) in ultra-pure water. OD – absorbance measured in optical density units. R^2– coefficient of determination.



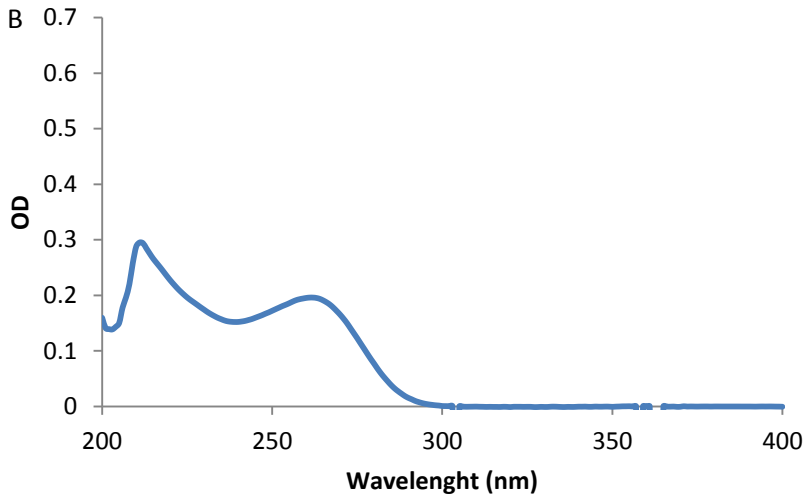


Figure 8: Absorption spectra of the highest cephalexin concentration (10 mg/l) in artificial sea water at 0 h (A) and 96 h (B).

After 96 h of their preparation in experimental conditions similar to those used in further bioassays (temperature 20 ± 1ºC; photoperiod 8 h L: 16 h D), the spectra of Figure 8A solutions are shown in Figure 8B. The general pattern is similar suggesting no significant degradation of cephalexin into other substances. At 260 nm, the intensity of the peaks in ASW at 0h and 96h was 0.214 and 0.199, respectively, indicating a similar pattern. These findings suggest no degradation of cephalexin during 96 h in our experimental conditions.

3.2. Spectrofluorometry method to assess MP concentrations

A positive and significant correlation ($N = 24$, $r = 0.989$, $p < 0.05$) between the fluorescence units and the nominal MP concentrations was found in ASW. The linear regression model adjusted to the calibration curve, using fluorescence (fluorescence units) as independent variable and MP concentration (mg/l) as dependent variable, was: MP concentration (mg/l) = - 0.1295 + 0.0134 x fluorescence (F units) (Figure 9). Overall, these findings indicate that this spectrofluorometry method can be used to measure the concentrations of MP in the test media in the range of concentrations tested.

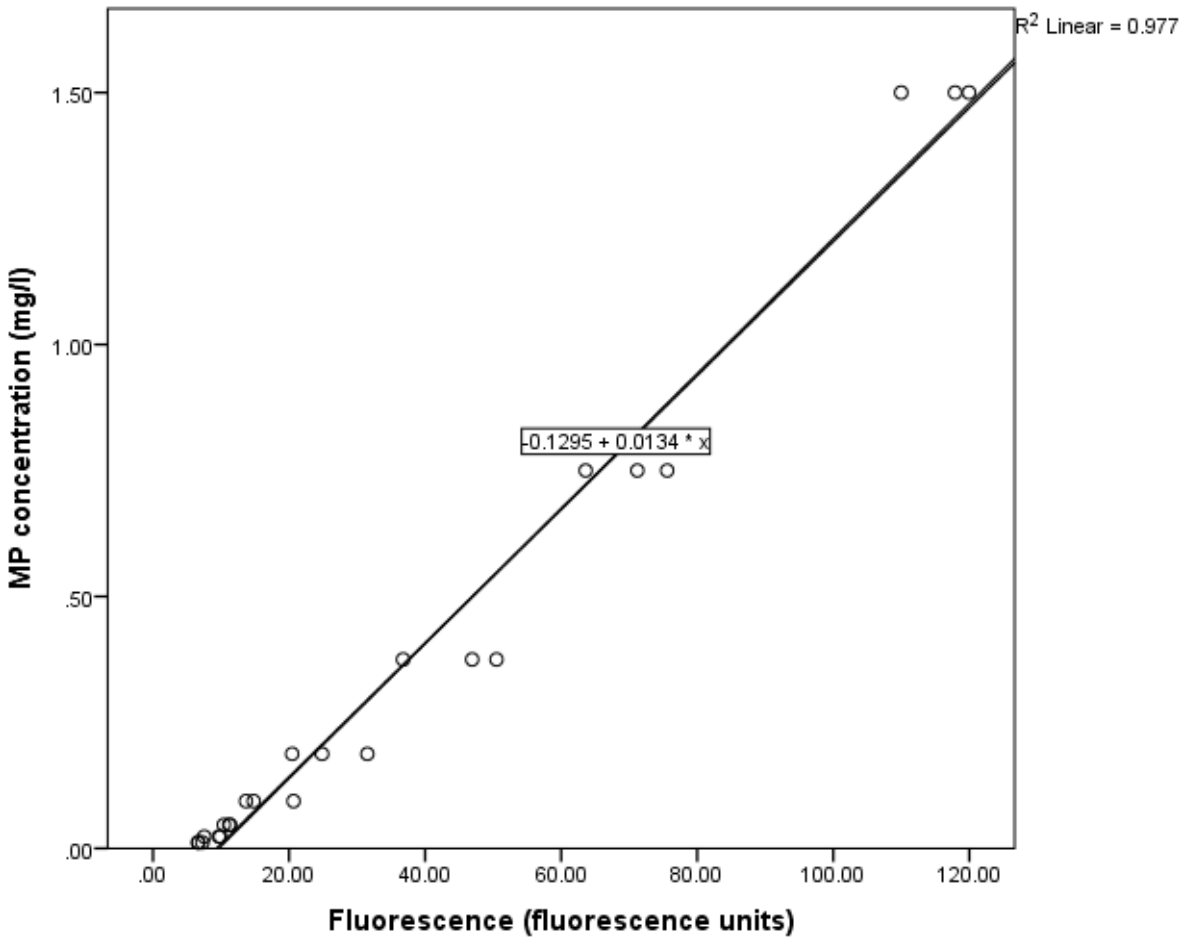


Figure 9: Linear model fitted to the calibration curve (MP fluorescence units versus nominal MP concentrations in artificial sea water. R^2 – coefficient of determination.

3.3. Optimization of the conditions for use in the bioassays

The mean of the physico-chemical parameters monitored along the first bioassay are indicated in Table 1. The variation of pH was always lower than 1 pH unit, and the water concentration of oxygen was higher than 60% of the air saturation value. No mortality was recorded in any of the treatments. Therefore, the validity criteria of the OCDE guideline for acute testing with juvenile fish (OECD, 1992) were fulfilled except regarding the temperature. The variation of this parameter was higher (from 15.7ºC to 19.6ºC) because a semi-natural temperature variation was considered.

Table 1: Physico-chemical parameters monitored in the test media along the 96 h of the first bioassay. The values of each parameter are the mean of six measurements made in individual test beakers per treatment with the respective standard error of the mean.

Parameters	Treatment	0h	24h	48h	72h	96h
Temperature (ºC)	**Control**	19.1 ± 0.08	18.4 ± 0.35	17.9 ± 0.29	18.3 ± 0.27	16.1 ± 0.33
	0.156	19.3 ± 0.05	18.4 ± 0.10	17.9 ± 0.08	18.4 ± 0.06	16.5 ± 0.06
	0.313	19.3 ± 0.06	18.0 ± 0.12	17.5 ± 0.11	18.1 ± 0.13	16.0 ± 0.14
	0.625	19.5 ± 0.04	18.2 ± 0.11	17.8 ± 0.08	18.3 ± 0.05	16.2 ± 0.06
	1.25	19.5 ± 0.04	18.3 ± 0.06	17.9 ± 0.05	18.5 ± 0.06	16.3 ± 0.11
	2.5	19.5 ± 0.04	18.2 ± 0.07	17.9 ± 0.05	18.4 ± 0.08	16.2 ± 0.06
	5	19.5 ± 0.04	18.2 ± 0.07	17.8 ± 0.06	18.4 ± 0.06	16.3 ± 0.07
pH	**Control**	8.54 ± 0.07	8.69 ± 0.05	8.73 ± 0.03	8.72 ± 0.03	8.74 ± 0.01
	0.156	8.68 ± 0.02	8.76 ± 0.01	8.75 ± 0.01	8.76 ± 0.01	8.73 ± 0.01
	0.313	8.69 ± 0.01	8.76 ± 0.01	8.75 ± 0.01	8.77 ± 0.01	8.75 ± 0.00
	0.625	8.69 ± 0.00	8.77 ± 0.01	8.75 ± 0.01	8.78 ± 0.01	8.74 ± 0.00
	1.25	8.69 ± 0.00	8.76 ± 0.01	8.74 ± 0.00	8.76 ± 0.01	8.73 ± 0.00
	2.5	8.69 ± 0.01	8.76 ± 0.01	8.75 ± 0.00	8.77 ± 0.01	8.73 ± 0.00
	5	8.86 ± 0.17	8.77 ± 0.00	8.76 ± 0.00	8.79 ± 0.00	8.73 ± 0.00
O_2 dissolved (mg/l)	**Control**	7.44 ± 0.01	7.64 ± 0.06	7.74 ± 0.06	7.57 ± 0.08	8.09 ± 0.07
	0.156	7.45 ± 0.01	7.63 ± 0.02	7.69 ± 0.02	7.53 ± 0.03	7.98 ± 0.02
	0.313	7.46 ± 0.01	7.65 ± 0.06	7.78 ± 0.02	7.57 ± 0.07	8.07 ± 0.04
	0.625	7.48 ± 0.02	7.63 ± 0.03	7.72 ± 0.02	7.58 ± 0.02	8.04 ± 0.02
	1.25	7.49 ± 0.01	7.59 ± 0.02	7.70 ± 0.02	7.49 ± 0.05	8.00 ± 0.04
	2.5	7.47 ± 0.02	7.65 ± 0.02	7.73 ± 0.01	7.61 ± 0.03	8.07 ± 0.02
	5	7.47 ± 0.01	7.67 ± 0.02	7.74 ± 0.01	7.61 ± 0.02	8.07 ± 0.01

Table 2: Nominal and mean actual concentration of cephalexin and cephalexin decay (%) recorded during the first bioassay. The values are expressed as means ± standard errors of the mean. The S.E.M. are within brackets. Actual cephalexin concentrations were calculated from the individual readings using the linear model: actual cephalexin concentration (mg/l) = - 0.015 + 46.521 x O.D. Deviation (%) relatively to nominal concentrations = 100 – (actual concentration x 100 / nominal concentration). Decay (%) = (mean 0 h absorbance - mean 96 h absorbance) x 100 / mean 0 h absorbance. N – Number of samples analyzed. O.D. – optical density units.

Nominal cephalexin concentrations (mg/l)	**N**	**0h mean (±S.E.M) absorbance readings (O.D.)**	**Mean (±S.E.M) actual cephalexin concentrations (mg/l)**	**Mean deviation (%)**	**96h mean (±S.E.M) absorbance readings (O.D.)**	**Decay (%)**
0.156	6	0.002 (± 0.001)	0.094 (± 0.020)	40.0	0.001 (± 0.000)	57
0.313	6	0.005 (± 0.001)	0.233 (± 0.040)	25.5	0.002 (± 0.000)	22
0.625	6	0.013 (± 0.001)	0.598 (± 0.052)	4.40	0.009 (± 0.001)	24
1.25	6	0.024 (± 0.001)	1.11 (± 0.023)	11.3	0.020 (± 0.001)	16
2.5	6	0.053 (± 0.001)	2.43 (± 0.046)	2.90	0.045 (± 0.001)	15
5	6	0.106 (± 0.002)	4.91 (± 0.073)	1.83	0.088 (± 0.002)	12

The actual concentrations of cephalexin in test media at the beginning of the bioassay, estimated from the linear model of Figure 6 from the absorbance of test media are shown in Table 2. Except for the two lowest treatments, the % of deviation of actual concentrations relatively to nominal ones was lower than 20%. Thus, according the OECD guideline nº 203 (OECD, 1992), in these particular cases, further results can be expressed in function of nominal concentrations. It should be noted that at the lowest concentrations tested, the sensitivity of the method may be low, as suggest by the very low absorbance values and the higher departure from the nominal concentrations relatively to the variation calculated for higher treatments. At 96 h, at concentrations between 1.25 and 5, the decay did not exceed 20% and thus it can be considered that no significant decay occur during the bioassay (OECD, 1992). However, 57% was estimated

for the lowest concentration, thus suggesting low sensitivity of the method at low concentrations as previously indicated.

The total length and weight of the fish measured at the end of the exposure period are indicated in Table 3. The overall mean and the standard error of the mean (S.E.M.) of the total length and weight were 1.92 ± 0.185 cm and 0.080 ± 0.025 g, respectively. For both parameters, no significant differences among treatments were found (total length: $F_{6,35}$ = 0.634, p > 0.05; total weight: $F_{6,35}$= 0.535, p > 0.05).

Table 3: Total length and weight of the fish measured after the 96 h bioassay. Results are expressed as the mean of six fish per treatment with the respective standard error of the mean.

	Treatments						
	Control	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**
Length (cm)	2.0 ± 0.147	1.9 ± 0.183	1.9 ± 0.273	1.9 ± 0.186	1.9 ± 0.234	2.0 ± 0.164	1.9 ± 0.105
Weight (g)	0.098 ± 0.019	0.077 ± 0.026	0.082 ± 0.038	0.076 ± 0.020	0.083 ± 0.033	0.075 ± 0.023	0.071 ± 0.017

Table 4: Statistical results of the 1–ANOVA obtained for all the endpoints tested. N – Number of fish analyzed. *df* – degrees of freedom; Sig. – level of significance.

Endpoint	**N**	***df***	**F**	**p**
Predatory performance	42	6, 35	2.815	0.024
AChE	42	6, 35	21.776	0.000
GST	42	6, 35	0.863	0.531
LPO	42	6, 35	0.580	0.743

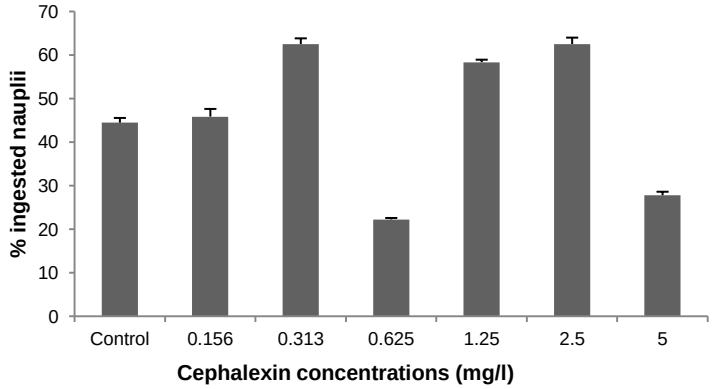


Figure 10: Predatory performance of *Pomatoschistus microps* juveniles assessed individually after 96 h of exposure to cephalexin (0.156, 0.313, 0.625, 1.25, 2.5 and 5 mg/l). Six fish were used per treatment. Results are the mean of the percentage of ingested nauplii relatively to the total number of prey offered to the fish (12) with corresponding standard error bars.

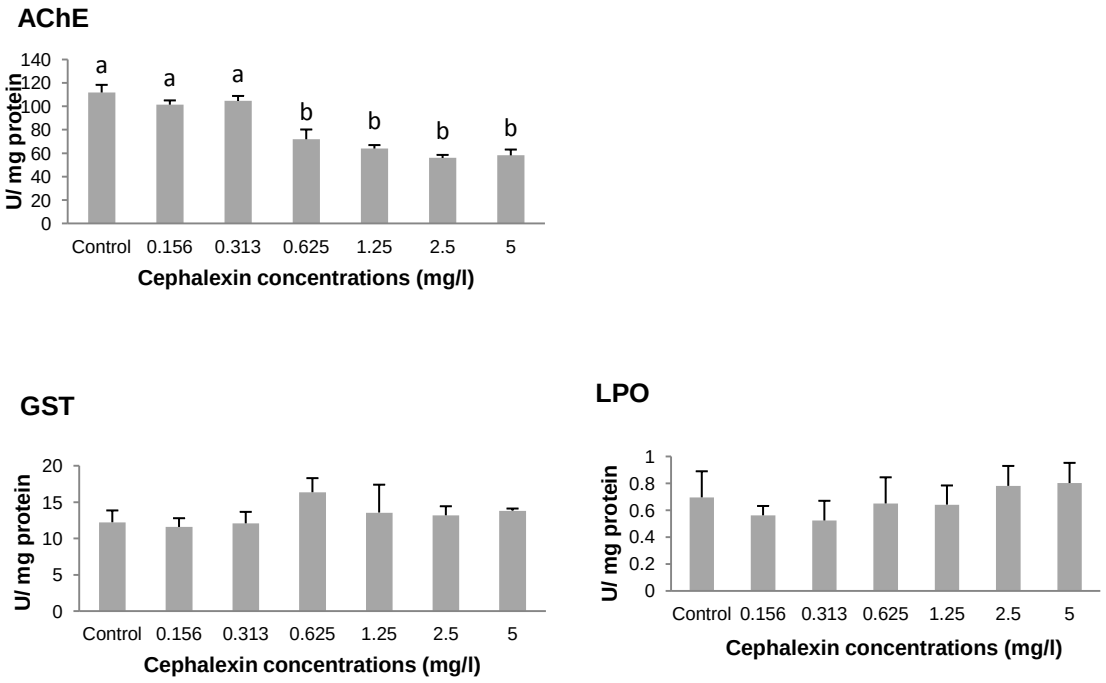


Figure 11: Effects of cephalexin (0.156, 0.313, 0.625, 1.25, 2.5 and 5 mg/l) on the activity of acetylcholinesterase (AChE) and glutathione-S-transferases (GST) enzymes and lipid peroxidation levels (LPO) of *Pomatoschistus microps* juveniles after 96 h of exposure. The results are the mean of six fish with corresponding standard error bars. Different letters above the bars indicate statistically significant differences (1-ANOVA and Tukey's multi-comparison test). U – nmol/min for AChE and GST activity and nmol of TBARS/min for LPO levels.

The effects of cephalexin on the predatory performance of *P. microps* juveniles are shown in Figure 10. Significant differences among treatments were found (Table 4), but the Tukey test was not able to discriminate statistically significant differences.

Cephalexin significantly reduced the AChE activity at concentrations equal or higher than 0.625 mg/l, reaching a maximum of 50% relatively to the control group at 2.5 mg/l. Thus, these preliminary findings suggest an anti-cholinesterase effect of cephalexin. In relation to GST activity and LPO levels, no significant differences were found. Overall, the results of this bioassay indicate that the highest cephalexin concentrations tested may induce toxic effects on *P. microps* juveniles. However, the determination of the predatory performance seems to have had some problems because no clear dose-response was observed.

3.4. Preliminary bioassay to assess the effects of cephalexin

The mean of the physico-chemical parameters monitored along the preliminary bioassay are indicated in Table 5. The variation of pH was always lower than 1 pH unit, and the water concentration of oxygen was always higher than 60% of the air saturation value. The variation of temperature was less than 1ºC. No mortality was recorded in any of the treatments. Therefore, the validity criteria of the OCDE guideline for acute testing with juvenile fish (OECD, 1992) were achieved relatively to these parameters.

Table 5: Physico-chemical parameters monitored in the test media along the 96 h of the preliminary bioassay. The values of each parameter are the mean of nine measurements made in individual test beakers per treatment with the respective standard error of the mean.

Parameters	Treatment	0h	24h	48h	72h	96h
Temperature (ºC)	**Control**	21.4 ± 0.18	20.6 ± 0.15	20.9 ± 0.05	21.3 ± 0.08	20.7 ± 0.07
	0.313	21.4 ± 0.16	20.6 ± 0.18	20.6 ± 0.20	21.4 ± 0.12	20.8 ± 0.21
	0.625	21.2 ± 0.11	20.7 ± 0.15	20.5 ± 0.16	21.1 ± 0.21	21.0 ± 0.19
	1.25	21.3 ± 0.39	21.5 ± 0.63	20.9 ± 0.07	21.3 ± 0.07	21.2 ± 0.06
	2.5	20.6 ± 0.06	20.9 ± 0.05	20.5 ± 0.04	21.5 ± 0.09	21.3 ± 0.13
	5	20.5 ± 0.08	20.7 ± 0.08	21.1 ± 0.06	21.4 ± 0.13	21.2 ± 0.10

Continuation Table 5

pH	**Control**	8.46 ± 0.03	8.45 ± 0.06	8.50 ± 0.04	8.52 ± 0.03	8.50 ± 0.04
	0.313	8.55 ± 0.01	8.50 ± 0.01	8.58 ± 0.01	8.55 ± 0.01	8.57 ± 0.00
	0.625	8.54 ± 0.01	8.53 ± 0.02	8.54 ± 0.02	8.50 ± 0.03	8.52 ± 0.02
	1.25	8.50 ± 0.03	8.51 ± 0.02	8.56 ± 0.01	8.59 ± 0.01	8.58 ± 0.00
	2.5	8.51 ± 0.01	8.55 ± 0.00	8.57 ± 0.00	8.56 ± 0.01	8.55 ± 0.01
	5	8.51 ± 0.01	8.54 ± 0.00	8.56 ± 0.01	8.59 ± 0.01	8.57 ± 0.01
O_2 dissolved (mg/l)	**Control**	8.44 ± 0.06	8.80 ± 0.02	8.12 ± 0.04	8.48 ± 0.02	8.17 ± 0.03
	0.313	8.45 ± 0.08	8.78 ± 0.06	8.89 ± 0.07	8.33 ± 0.04	8.92 ± 0.08
	0.625	8.49 ± 0.06	8.38 ± 0.05	8.89 ± 0.03	8.07 ± 0.04	8.83 ± 0.10
	1.25	8.41 ± 0.08	8.64 ± 0.13	8.95 ± 0.03	8.97 ± 0.02	8.78 ± 0.01
	2.5	8.81 ± 0.02	8.29 ± 0.12	8.87 ± 0.02	8.84 ± 0.05	8.89 ± 0.04
	5	8.79 ± 0.03	8.68 ± 0.03	8.59 ± 0.05	8.93 ± 0.03	8.87 ± 0.04

Table 6: Nominal and mean actual concentration of cephalexin and cephalexin decay (%) recorded during the preliminary bioassay. The values are expressed as means ± standard errors of the mean. The S.E.M. are within brackets. Actual cephalexin concentrations were calculated from the individual readings using the linear model: actual cephalexin concentration (mg/l) = - 0.015 + 46.521 x O.D. Deviation (%) relatively to nominal concentrations = 100 – (actual concentration x 100 / nominal concentration). Decay (%) = (mean 0 h absorbance - mean 96 h absorbance) x 100 / mean 0 h absorbance. N – Number of samples analyzed. O.D. – optical density units.

Nominal cephalexin concentrations (mg/l)	**N**	**0h mean (±S.E.M) absorbance readings (O.D.)**	**Mean (±S.E.M) actual cephalexin concentrations (mg/l)**	**Mean deviation (%)**	**96h mean (±S.E.M) absorbance readings (O.D.)**	**Decay (%)**
0.313	9	0.008 (±0.001)	0.394 (± 0.028)	26.0	0.007 (±0.002)	24
0.625	9	0.015 (±0.002)	0.684 (± 0.049)	9.42	0.013 (±0.002)	12
1.25	9	0.032 (±0.002)	1.47 (± 0.101)	17.9	0.029 (±0.002)	10
2.5	9	0.062 (±0.004)	2.87 (± 0.149)	15.0	0.054 (±0.002)	14

Continuation Table 6

5	9	0.124 (±0.004)	5.74 (± 0.180)	14.7	0.104 (±0.004)	15

The actual concentrations of cephalexin in test media at the beginning of the bioassay, estimated from the linear model of Figure 6 from the absorbance of test media are shown in Table 6. Except for the lowest treatment, the % of deviation of actual concentrations relatively to nominal ones was lower than 20%. Thus, according the OECD guideline nº 203 (OECD, 1992), further results can be expressed in function of nominal concentrations. However, at 0.313 mg/l, the deviation was higher than 20%. Considering the very low absorbance at this concentration this suggests low sensitivity of the spectrophotometry method at this concentration. At 96 h, at concentrations between 0.625 and 5, the decay did not exceed 20% and thus it can be considered that no significant decay occur during the bioassay (OECD, 1992). However, 24% was estimated for the lowest concentration, a problem that may be due to low sensitivity of the method at the lowest concentration of cephalexin tested.

The total length and weight of the fish measured at the end of the exposure period are indicated in Table 7. The overall mean and the standard error of the mean (S.E.M.) of the total length and weight were 2.1 ± 0.196 cm and 0.089 ± 0.025 g, respectively. For both parameters, no significant differences among treatments were found (total length: $F_{5,48}$= 0.707, p > 0.05; total weight: $F_{5, 48}$= 0.434, p > 0.05).

Table 7: Total length and weight of the fish measured after the 96 h bioassay. Results are expressed as mean of nine fish per treatment with the respective standard error of the mean.

	Treatments					
	Control	**0.313**	**0.625**	**1.25**	**2.5**	**5**
Length (cm)	2.1 ± 0.136	2.1 ± 0.250	2.2 ± 0.213	2.2 ± 0.274	2.0 ± 0.133	2.0 ± 0.142
Weight (g)	0.086 ± 0.016	0.084 ± 0.028	0.095 ± 0.023	0.104 ± 0.037	0.081 ± 0.022	0.089 ± 0.021

Table 8: Statistical results of the 1-ANOVA obtained for all the endpoints tested. N – Number of fish analysed, except for EROD where pooled samples (3 fish) are shown. *df* – degrees of freedom; Sig. – level of significance.

Endpoint	N	*df*	F	p
Predatory performance	54	5, 48	7.318	0.000
AChE	54	5, 48	4.016	0.004
EROD	18	5, 12	0.685	0.644
GST	54	5, 48	0.649	0.663
LPO	54	5, 48	0.560	0.730

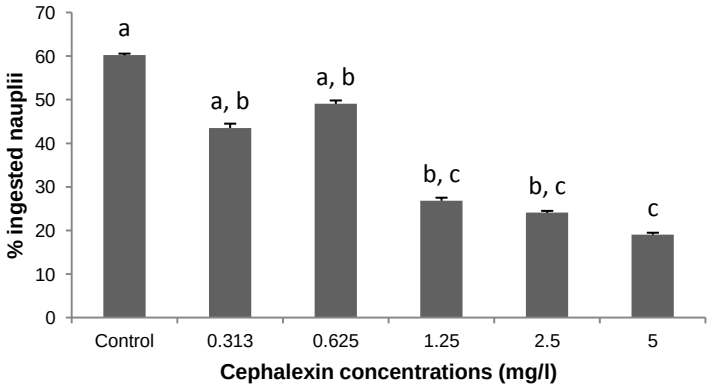


Figure 12: Predatory performance of *Pomatoschistus microps* assessed individually after 96 h of exposure to cephalexin (0.313, 0.625, 1.25, 2.5 and 5 mg/l). Nine fish were used per treatment. Results are expressed as the mean of the percentage of ingested nauplii relatively to the total number offered (12) with corresponding S.E.M bars. Different letters above the bars indicate statistically significant differences (1-ANOVA and Tukey's multi-comparison test).

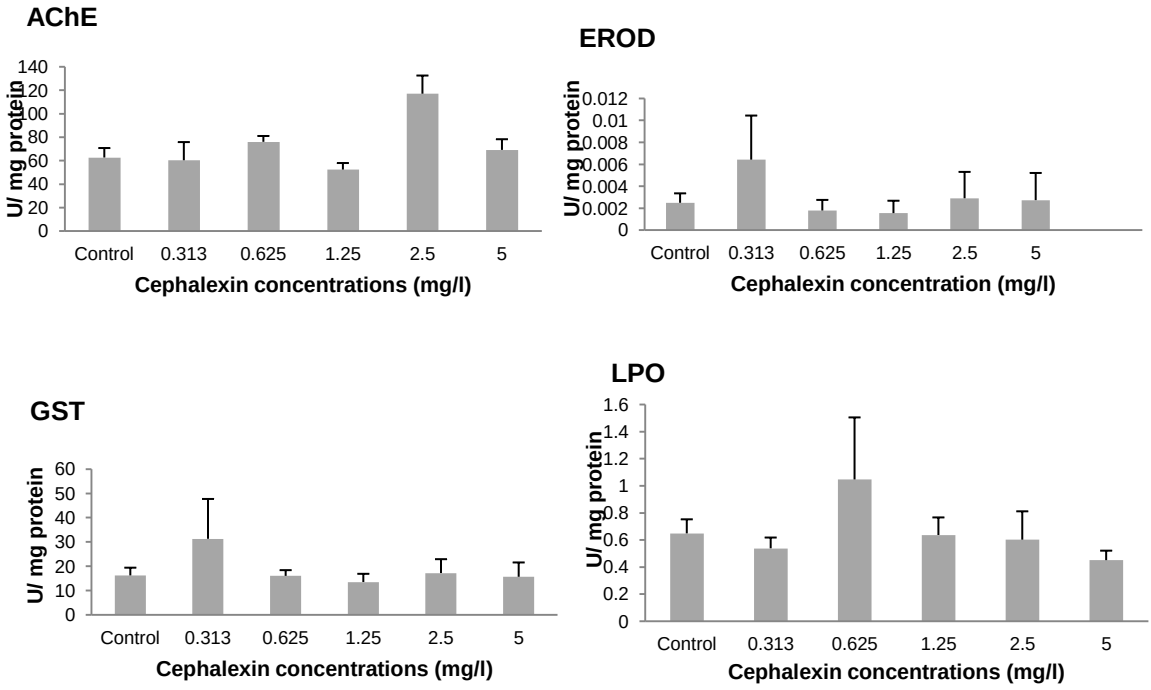


Figure 13: Effects of cephalexin (0.313, 0.625, 1.25, 2.5 and 5 mg/l) on the activity of acetylcholinesterase (AChE), ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferases (GST) enzymes and lipid peroxidation levels (LPO) of *Pomatoschistus microps* juveniles after 96 h of exposure. The results are the mean of nine fish with corresponding standard error bars. U – nmol/min for AChE and GST activity, pmol/min for EROD activity and nmol of TBARS/min for LPO levels.

The effects of cephalexin on the predatory performance of *P. microps* juveniles are shown in Figure 12. Significant differences among treatments were found (Table 8). Concentrations of cephalexin equal or higher than 1.25 mg/l caused a significant reduction of the predatory performance of the fish by 32 - 45%. The effects of cephalexin on AChE activity are shown in Figure 13 and the results of the statistical analysis in Table 8. Contrary to the inhibition found in the training bioassay, no clear pattern was found. Significant differences among treatments were found (Table 8). No significant differences were found for EROD and GST activity and for LPO levels (Table 8).

Based on the results of this preliminary bioassay it was decided to adjust the range of concentrations to be tested in further bioassays: between 1.25 and 10 mg/l.

3.5. Influence of microplastics on the toxicity of cephalexin assessed at 20ºC

The mean of the physico-chemical parameters monitored along the bioassay performed at 20ºC are indicated in Table 9. The variation of pH was always lower than 1 pH unit, and the water concentration of oxygen was always higher than 60% of the air saturation value. The variation of temperature was less than 1ºC. No mortality higher than 10% was recorded in the control group. Therefore, the validity criteria of the OCDE guideline for acute testing with juvenile fish (OECD, 1992) regarding these parameters were fulfilled.

Table 9: Physico-chemical parameters monitored in the test media along the 96 h of the bioassay performed at 20ºC. The values of each parameter are the mean of ten to twelve measurements made in individual test beakers per treatment with the respective standard error of the mean.

Parameters	Treatment	0h	24h	48h	72h	96h
Temperature (ºC)	**Control**	20.4 ± 0.05	20.3 ± 0.12	20.3 ± 0.14	20.3 ± 0.08	20.1 ± 0.14
	MP	20.4 ± 0.16	20.2 ± 0.09	20.4 ± 0.18	20.4 ± 0.18	20.3 ± 0.17
	1.25	20.2 ± 0.18	20.4 ± 0.07	20.1 ± 0.21	19.8 ± 0.22	20.0 ± 0.09
	2.5	20.3 ± 0.09	20.5 ± 0.12	20.1 ± 0.22	20.3 ± 0.09	20.0 ± 0.06
	5	20.1 ± 0.15	20.6 ± 0.14	20.3 ± 0.07	19.9 ± 0.14	20.3 ± 0.17
	10	20.0 ± 0.05	20.4 ± 0.08	20.2 ± 0.06	20.4 ± 0.17	19.9 ± 0.18
	1.25+MP	20.4 ± 0.17	20.4 ± 0.07	20.4 ± 0.04	20.4 ± 0.18	19.9 ± 0.09
	2.5+MP	20.4 ± 0.19	20.4 ± 0.16	20.4 ± 0.09	20.2 ± 0.19	20.4 ± 0.11
	5+MP	20.3 ± 0.28	19.9 ± 0.11	20.2 ± 0.07	19.9 ± 0.21	20.2 ± 0.12
	10+MP	20.4 ± 0.31	19.8 ± 0.13	19.9 ± 0.10	20.1 ± 0.19	20.3 ± 0.10
pH	**Control**	8.67 ± 0.01	8.78 ± 0.09	8.67 ± 0.02	8.73 ± 0.02	8.59 ± 0.01
	MP	8.71 ± 0.01	8.73 ± 0.01	8.72 ± 0.01	8.84 ± 0.08	8.59 ± 0.02
	1.25	8.69 ± 0.00	8.73 ± 0.01	8.73 ± 0.02	8.76 ± 0.01	8.62 ± 0.03
	2.5	8.73 ± 0.01	8.67 ± 0.04	8.79 ± 0.08	8.74 ± 0.01	8.61 ± 0.00
	5	8.73 ± 0.01	8.68 ± 0.10	8.67 ± 0.01	8.71 ± 0.01	8.76 ± 0.01
	10	8.51 ± 0.01	8.75 ± 0.00	8.73 ± 0.00	8.75 ± 0.01	8.62 ± 0.01
	1.25+MP	8.53 ± 0.02	8.68 ± 0.01	8.73 ± 0.01	8.80 ± 0.01	8.64 ± 0.00
	2.5+MP	8.51 ± 0.01	8.66 ± 0.00	8.74 ± 0.02	8.78 ± 0.02	8.63 ± 0.02
	5+MP	8.63 ± 0.04	8.64 ± 0.02	8.73 ± 0.00	8.73 ± 0.01	8.65 ± 0.01
	10+MP	8.56 ± 0.03	8.71 ± 0.01	8.73 ± 0.01	8.74 ± 0.01	8.65 ± 0.00

Continuation Table 9

O_2 dissolved (mg/l)	**Control**	8.61 ± 0.05	8.92 ± 0.04	9.28 ± 0.07	9.88 ± 0.02	9.08 ± 0.04
	MP	8.97 ± 0.05	9.08 ± 0.05	9.31 ± 0.06	9.87 ± 0.01	9.19 ± 0.06
	1.25	8.59 ± 0.06	9.19 ± 0.05	9.48 ± 0.02	9.69 ± 0.02	9.40 ± 0.04
	2.5	8.86 ± 0.06	9.22 ± 0.09	9.37 ± 0.02	9.27 ± 0.03	9.40 ± 0.02
	5	8.67 ± 0.06	9.53 ± 0.07	9.43 ± 0.06	9.56 ± 0.04	9.45 ± 0.06
	10	8.78 ± 0.07	9.74 ± 0.01	9.59 ± 0.02	8.93 ± 0.02	9.60 ± 0.02
	1.25+MP	8.74 ± 0.04	9.37 ± 0.02	9.34 ± 0.02	9.18 ± 0.01	9.25 ± 0.05
	2.5+MP	8.71 ± 0.02	9.24 ± 0.01	9.45 ± 0.01	8.98 ± 0.03	9.32 ± 0.06
	5+MP	8.67 ± 0.03	9.56 ± 0.04	9.56 ± 0.03	9.29 ± 0.02	9.19 ± 0.05
	10+MP	8.67 ± 0.01	9.76 ± 0.03	9.21 ± 0.03	9.19 ± 0.03	9.32 ± 0.04

Table 10: Nominal and mean actual concentration of cephalexin and cephalexin decay (%) recorded during the bioassay performed at 20ºC. The values are expressed as means ± standard errors of the mean. The S.E.M. are within brackets. Actual cephalexin concentrations were calculated from the individual readings using the linear model: actual cephalexin concentration (mg/l) = - 0.015 + 46.521 x O.D. Deviation (%) relatively to nominal concentrations = 100 – (actual concentration x 100 / nominal concentration). Decay (%) = (mean 0 h absorbance - mean 96 h absorbance) x 100 / mean 0 h absorbance. N – Number of samples analyzed. O.D. – optical density units.

Nominal cephalexin concentrations (mg/l)	**N**	**0h mean (±S.E.M) absorbance readings (O.D.)**	**Mean (±S.E.M) actual cephalexin concentrations (mg/l)**	**Mean deviation (%)**	**96h mean (±S.E.M) absorbance readings (O.D.)**	**Decay (%)**
1.25	12	0.025 (± 0.002)	1.15 (± 0.096)	8.13	0.020 (± 0.002)	20
2.5	12	0.054 (± 0.002)	2.47 (± 0.089)	1.01	0.045 (± 0.002)	16
5	10	0.102 (± 0.005)	4.73 (± 0.222)	5.44	0.091 (± 0.001)	10
10	11	0.196 (± 0.004)	9.10 (± 0.169)	9.04	0.189 (± 0.003)	4
1.25+MP	12	0.032 (± 0.002)	1.45 (± 0.129)	16.1	0.025 (± 0.001)	20
2.5+MP	12	0.053 (± 0.002)	2.46 (± 0.103)	1.73	0.044 (± 0.001)	17
5+MP	11	0.106 (±0.002)	4.90 (± 0.114)	1.98	0.093 (±0.002)	12
10+MP	10	0.206 (±0.006)	9.57 (± 0.301)	4.27	0.194 (±0.002)	6

Table 11: Nominal and mean actual concentration of MP and MP decay (%) recorded during the bioassay performed at 20ºC. The values are expressed as means ± standard errors of the mean. The S.E.M. are within brackets. Actual MP concentrations were calculated from the individual fluorescence readings using the linear model: actual MP concentration (mg/l) = - 0.1295 + 0.013 x fluorescence (F units). Deviation (%) relatively to nominal concentrations = 100 – (actual MP concentration x 100 /0.184); % Decay = (mean 0 h absorbance - mean 96 h absorbance) x 100 / mean 0 h absorbance. Conc. – concentrations. N – Number of samples analyzed. Nominal concentrations were 0.184 mg/l.

Nominal cephalexin conc. (mg/l)	Nominal MP conc. (mg/l)	N	0h mean (± S.E.M.) fluorescence readings (F units)	Mean (± S.E.M.) actual MP conc. (mg/l)	Deviation (%)	96h mean (± S.E.M.) fluorescence readings (F units)	MP decay (%)
0	0.184	11	21.76 (±0.708)	0.162 (±0.009)	12	16.97 (±0.568)	22
1.25	0.184	12	23.55 (±0.809)	0.186 (±0.011)	3	16.81 (±0.851)	29
2.5	0.184	12	22.53 (±0.671)	0.173 (±0.009)	6	16.23 (±0.533)	28
5	0.184	11	22.71 (±0.588)	0.185 (±0.012)	0	16.02 (±0.540)	30
10	0.184	10	23.13 (±0.980)	0.180 (±0.002)	7	16.97 (±0.973)	27

The actual concentrations of cephalexin in test media at the beginning of the bioassay, estimated from the linear model of Figure 6 from the absorbance of test media are shown in Table 10. In all treatments, the % of deviation of actual concentrations relatively to nominal ones was lower than 20%. Thus, according the OECD guideline nº 203 (OECD, 1992), further results can be expressed in function of nominal concentrations. The decay of cephalexin in test media was always equal or lower than 20%, thus it can be considered that no significant decay occurs during the bioassay (OECD, 1992). The actual MP concentrations calculated from the linear model of Figure 9 are shown in Table 11.

The deviations of the actual concentrations relatively to the nominal one (0.184 mg/l) were lower than 20%, and thus results are further indicated relatively to nominal concentrations. At 96 h, the decay of MP during the bioassay was higher than 20%, indicating a significant decay of the test substance during the bioassay. This suggests that MP may aggregate and precipitate, not excluding other possibilities. It is interesting to note that the decay of MP in the presence of cephalexin (between 27 and 30%) was highest than the decay of MP when present alone in the test media (22%). This suggests interactions between MP and cephalexin.

Table 12: Mortality (%) recorded in different treatments after 96 h of exposure to cephalexin alone and in the presence of MP at 20ºC. MP – microplastics present in test media (0.184 mg/l).

	Treatments (mg/l)									
	Control	**MP only**	**1.25**	**2.5**	**5**	**10**	**1.25+MP**	**2.5+MP**	**5+MP**	**10+MP**
Mortality (%)	8	8	0	0	16	8	0	0	8	16

The total length and weight of the fish measured at the end of the exposure period are indicated in Table 13. The overall mean and the standard error of the mean (S.E.M.) of the total length and weight were 2.6 ± 0.236 cm and 0.186 ± 0.049 g, respectively. For both parameters, no significant differences among treatments were found (total length: $F_{9,102}$= 0.230, p > 0.05; total weight: $F_{9,102}$= 0.655, p > 0.05).

Table 13: Total length and weight of the fish measured after the 96 h bioassay. Results are expressed as the mean of ten to twelve fish per treatment with the respective standard error of the mean.

Treatments									
Control	**1.25**	**2.5**	**5**	**10**	**MP**	**1.25+MP**	**2.5+MP**	**5+MP**	**10+MP**
2.6 ± 0.219	2.6 ± 0.147	2.4 ± 0.247	2.5 ± 0.216	2.6 ± 0.228	2.6 ± 0.270	2.5 ± 0.243	2.5 ± 0.229	2.5 ± 0.280	2.7 ± 0.253
0.182 ± 0.045	0.191 ± 0.033	0.171 ± 0.052	0.176 ± 0.061	0.193 ± 0.049	0.210 ± 0.057	0.179 ± 0.052	0.173 ± 0.049	0.185 ± 0.036	0.201 ± 0.058

Table 14: Summary of the statistical results obtained on the 2-ANOVA and the Tukey test ($p \leq 0.05$). Pred. perf. - Predatory performance (%). AChE – acetylcholinesterase activity (U/ mg protein). EROD - ethoxyresorufin-O-deethylase activity (U/ mg protein). GST – glutathione-S-transferases activity (U/ mg protein). LPO – lipid peroxidation levels (U/ mg protein). MP – presence of microplastics in test media (0.184 mg/l). CEP – cephalexin. When applied, different letters indicate statistically significant differences. *df* – degrees of freedom; Sig. – level of significance. S.E.M. – standard error of the mean.

Endpoint	Factor	Conc. (mg/l)	Mean ± S.E.M	Tukey test	F	p
Pred. perf.	Treatment				$F_{5, 102} = 5.290$	0.000
		Control	35.15 % ± 2.67	a		
		MP 0.184	26.97 % ± 4.35	a,b		
		CEP 1.25	23.19 % ± 1.89	b,c		
		CEP 2.5	21.53 % ± 1.89	b.c		
		CEP 5	21.27 % ± 1.80	b.c		
		CEP 10	17.30 % ± 1.57	c		
	MP	0	26.88 % ± 1.43		$F_{1, 102} = 1.941$	0.167
		0.184	20.95 % ± 1.28			
	Interaction				$F_{3, 102} = 1.993$	0.120
AChE	Treatment				$F_{5, 102} = 4.797$	0.001
		Control	53.15 U ± 8.17			
		MP 0.184	56.57 U ± 7.81			
		CEP 1.25	56.00 U ± 4.89			
		CEP 2.5	70.72 U ± 4.05)			
		CEP 5	71.15 U ± 4.25			
		CEP 10	72.15 U ± 4.86			
	MP	0	54.23 U ± 2.96		$F_{1, 102} = 30.100$	0.000
		0.184	75.37 U ± 2.62			
	Interaction				$F_{3, 102} = 0.549$	0.650

Continuation Table 14

EROD	Treatment	Control	0.05 U ± 0.01		$F_{5, 19} = 1.167$	0.361
		MP 0.184	0.14 U ± 0.07			
		CEP 1.25	0.04 U ± 0.01			
		CEP 2.5	0.04 U ± 0.01			
		CEP 5	0.07 U ± 0.02			
		CEP 10	0.03 U ± 0.01			
	MP	0	0.056 U ± 0.015		$F_{1, 19} = 5.872$	0.026
		0.184	0.051 U ± 0.008			
	Interaction				$F_{3, 19} = 0.445$	0.723
GST	Treatment	Control	7.37 U ± 0.69	a	$F_{5, 102} = 8.455$	0.000
		MP 0.184	9.86 U ± 0.95	a,b		
		CEP 1.25	15.30 U ± 1.95	b,c		
		CEP 2.5	13.57 U ± 1.73	b.c		
		CEP 5	15.44 U ± 2.58	b.c		
		CEP 10	17.04 U ± 1.40	c		
	MP	0	8.97 U ± 0.74		$F_{1, 102} = 115.502$	0.000
		0.184	18.97 U ± 1.10			
	Interaction				$F_{3, 102} = 13.881$	0.000

Continuation Table 14

LPO	Treatment			$F_{5, 102} = 4.280$	0.001
		Control	0.46 U ± 0.08		
		MP 0.184	0.34 U ± 0.08		
		CEP 1.25	0.63 U ± 0.18		
		CEP 2.5	0.56 U ± 0.07		
		CEP 5	0.46 U ± 0.04		
		CEP 10	0.67 U ± 0.07		
	MP	0	0.50 U ± 0.08		
		0.184	0.59 U ± 0.04	$F_{1, 102} = 13.608$	0.000
	Interaction			$F_{3, 102} = 1.533$	0.210

The results of cephalexin in the presence and absence of MP at 20ºC on distinct parameters of fish are shown in Figures 14 and 15, and the results of the 2-ANOVA are shown in Table 14. Concerning the predatory performance, significant differences among treatments, no significant differences induced by the presence of MP, and no significant interaction between treatments and MP presence were found (Table 14). Considering the overall means per treatment, cephalexin induced effects significantly different from the control group at concentrations equal or higher than 1.25 mg/l reaching 50% of predatory performance inhibition at the highest concentration tested. MP alone did not induce significant predatory performance differences relatively to the control group. The no significant interaction suggests that, at 20ºC the presence of MP did not interact with the effects of cephalexin on fish predatory performance.

Regarding AChE activity, significant differences among treatments, significant differences induced by the presence of MP and no significant interaction between treatments and MP presence were found (Table 14). Since there were differences in the presence and absence of MP, all the treatments were compared with 1-ANOVA ($F_{9, 102}$ = 6.521) and the Tukey test (Figure 15). The fish exposed to the treatment containing 5 mg/l of cephalexin and MP had higher AChE activity than those of the control group (≈ 60%), those exposed to MP alone (≈ 50%), and those exposed to 5 mg/l of cephalexin alone (≈ 52%). A similar situation happened regarding the fish exposed to 10 mg/l of cephalexin in the presence of MP (≈ 63% relatively to the control group). No significant effects were observed in fish only exposed to MP which is not in agreement with the results obtained

by Oliveira *et al.* (2013) where they found a decrease of AChE activity of fish exposed to 0.184 mg/l of MP. These results suggest toxicological interaction between cephalexin and MP on the activity of AChE on the fish.

For EROD activity, no significant differences among treatments, significant differences induced by the presence of MP and no significant interaction between treatments and MP presence were found (Table 14). Because differences in the presence of MP were observed, all the treatments were compared with 1-ANOVA ($F_{9, 19}$ = 1.205) and the Tukey test. However, results suggest no significant differences in the range of concentration tested. Furthermore, since 2-ANOVA found differences induced by MP, the interpretation of the results should be cautious.

With respect to GST activity, significant differences among treatments, significant differences induced by the presence of MP and significant interaction between treatments and the presence of MP were found, suggesting that MP interact with the effects of cephalexin (Table 14). The comparison of all the treatments with the 1-ANOVA ($F_{9, 102}$ = 21.148) and the Tukey test indicates: cephalexin alone induced an increase of GST activity but only at the highest concentration tested, whereas all the cephalexin concentrations on the presence of MP induced effects on the GST activity relatively to the control group (≈ 195, 168, 245 and 138% of induction at 1.25, 2.5, 5 and 10 mg/l of cephalexin and MP, respectively); MP alone had no significant effects. These results also suggest toxicological interaction between MP and cephalexin. Since MP alone had no significant effects on GST activity, cephalexin alone induced GST activity and in the presence of MP increased GST activity, these results suggest that the type of interaction may be potentiation. The induction of GST activity in the treatments where MP are present indicate that the biotransformation process is responding correctly. These findings may be of high interest to the fish fitness in their natural environment due to the crucial role that GST has on the detoxification process of xenobiotics and on the protection against oxidative stress.

Regarding LPO levels, significant differences among treatments, significant differences induced by the presence of MP and no significant interaction between treatments and MP presence were found (Table 14). Since there were differences in the presence and absence of MP, all the treatments were compared with 1-ANOVA ($F_{9, 102}$ = 3.738) and the Tukey test (Figure 15). The fish exposed to the treatment containing 2.5 mg/l of cephalexin and MP had higher LPO levels than those of the control group (≈ 43%), those exposed to MP alone (≈ 57%), and those exposed to 2.5 mg/l of cephalexin alone (≈ 59%). No significant effects were observed in fish only exposed to MP which is not in agreement with the results obtained by Oliveira *et al.* (2013). Although the presence of MP modifies the toxicity of cephalexin, the no significant interaction observed in the 2-ANOVA

suggest that MP did not interact with cephalexin on lipid peroxidation. Although the results are not significant, there is a tendency for the increase of LPO levels in the presence of MP, suggesting that the increase of GST observed was not enough against oxidative stress.

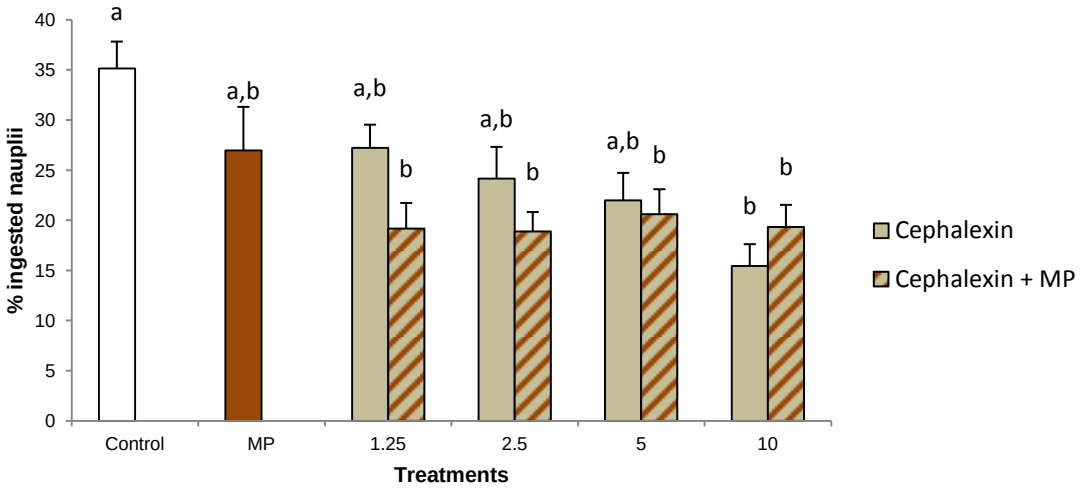
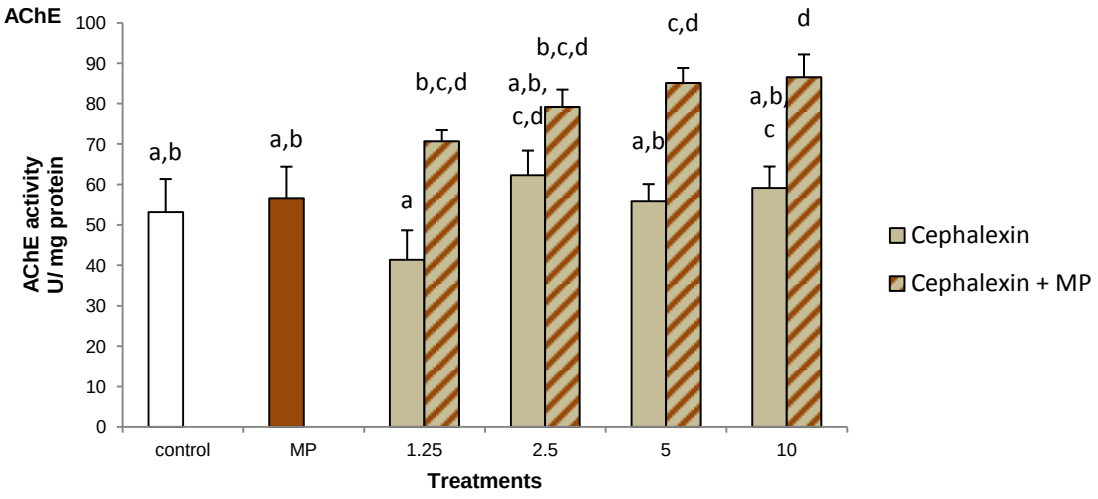
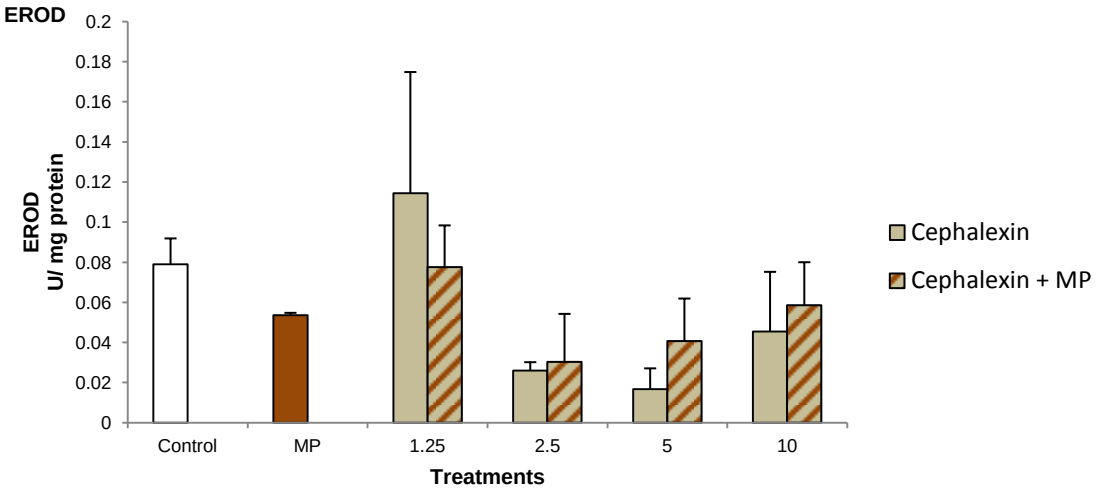
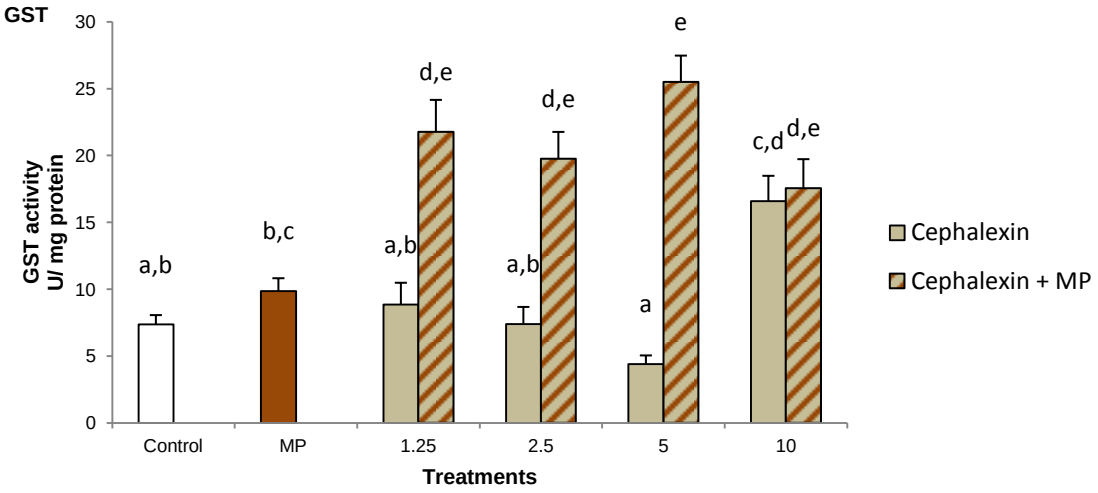


Figure 14: Predatory performance of *Pomatoschistus microps* assessed individually after 96 h of exposure to cephalexin alone (1.25, 2.5, 5 and 10 mg/l) and combined to microplastics (0.184 mg/l) and to microplastics alone (0.184 mg/l). Ten to twelve fish were used per treatment. Different letters above the bars indicate statistically significant differences (1-ANOVA and Tukey's multi-comparison test). Results are the mean of the percentage of ingested nauplii relatively to the total number offered (30) with corresponding S.E.M bars.




EROD
EROD U/ mg protein
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
MP
1.25
2.5
5
10
Treatments
Cephalexin
Cephalexin + MP


GST
GST activity U/ mg protein
30
25
20
15
10
5
0
a,b
b,c
a,b
d,e
a,b
d,e
a
e
c,d
d,e
Control
MP
1.25
2.5
5
10
Treatments
Cephalexin
Cephalexin + MP

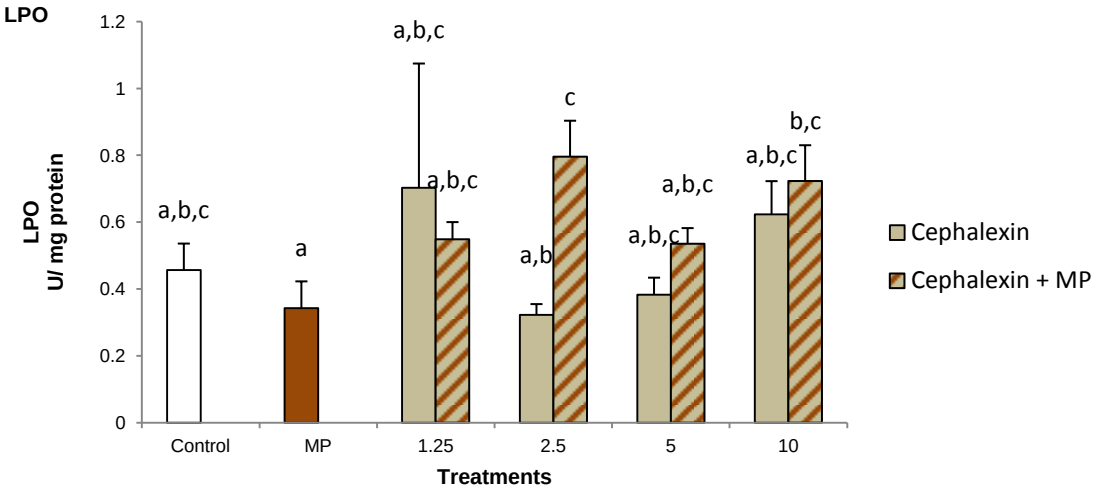


Figure 15: Effects of cephalexin alone (1.25, 2.5, 5 and 10 mg/l) and in combination of microplastics and effects of microplastics alone on the activity of acetylcholinesterase (AChE), ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferases (GST) enzymes and lipid peroxidation levels (LPO) of *Pomatoschistus microps* juveniles after 96 h of exposure. Different letters above the bars indicate statistically significant differences (1-ANOVA and Tukey's multi-comparison test). The results are the mean of ten to twelve fish with corresponding standard error bars. U – nmol/min for AChE and GST activity, pmol/min for EROD activity and nmol of TBARS/min for LPO levels.

3.6. Influence of microplastics on the toxicity of cephalexin assessed at 25ºC

The mean of the physico-chemical parameters monitored along the bioassay performed at 25ºC are indicated in Table 1. The variation of pH was always lower than 1 pH unit, and the water concentration of oxygen was higher than 60% of the air saturation value. The variation of temperature was less than 1ºC. No mortality higher than 10% was recorded in the control group. Therefore, the validity criteria of the OCDE guideline for acute testing with juvenile fish (OECD, 1992) regarding these parameters were fulfilled.

Table 15: Physico-chemical parameters monitored in the test media along the 96 h of the bioassay performed at 25ºC. The values of each parameter are the mean of eight to twelve measurements made in individual test beakers per treatment with the respective standard error of the mean.

Parameters	Treatment	0h	24h	48h	72h	96h
Temperature (ºC)	**Control**	24.7 ± 0.11	24.7 ± 0.10	24.7 ± 0.09	24.7 ± 0.11	25.2 ± 0.10
	MP	24.9 ± 0.15	24.7 ± 0.07	25.1 ± 0.09	24.8 ± 0.10	25.1 ± 0.09
	1.25	25.1 ± 0.18	24.6 ± 0.14	24.9 ± 0.07	25.0 ± 0.10	25.4 ± 0.07
	2.5	24.9 ± 0.18	24.8 ± 0.05	24.7 ± 0.13	25.0 ± 0.16	25.2 ± 0.04
	5	25.0 ± 0.14	24.6 ± 0.05	25.0 ± 0.15	25.1 ± 0.13	25.3 ± 0.09
	10	24.8 ± 0.14	24.7 ± 0.03	24.8 ± 0.13	25.4 ± 0.12	25.1 ± 0.15
	1.25+MP	25.2 ± 0.15	24.8 ± 0.07	24.8 ± 0.12	25.2 ± 0.16	25.3 ± 0.12
	2.5+MP	25.2 ± 0.16	24.9 ± 0.12	25.1 ± 0.11	25.2 ± 0.10	25.2 ± 0.07
	5+MP	25.3 ± 0.12	24.9 ± 0.07	25.3 ± 0.09	25.1 ± 0.12	25.1 ± 0.09
	10+MP	25.4 ± 0.11	24.7 ± 0.09	25.2 ± 0.07	25.1 ± 0.09	25.4 ± 0.16
pH	**Control**	8.74 ± 0.01	8.74 ± 0.02	8.77 ± 0.01	8.80 ± 0.01	8.79 ± 0.00
	MP	8.72 ± 0.02	8.76 ± 0.00	8.79 ± 0.00	8.81 ± 0.00	8.79 ± 0.01
	1.25	8.76 ± 0.00	8.77 ± 0.00	8.81 ± 0.00	8.81 ± 0.00	8.80 ± 0.00
	2.5	8.76 ± 0.00	8.77 ± 0.00	8.79 ± 0.02	8.81 ± 0.00	8.83 ± 0.01
	5	8.74 ± 0.01	8.76 ± 0.01	8.78 ± 0.01	8.82 ± 0.01	8.84 ± 0.02
	10	8.72 ± 0.01	8.76 ± 0.00	8.73 ± 0.00	8.80 ± 0.01	8.81 ± 0.01
	1.25+MP	8.73 ± 0.01	8.76 ± 0.00	8.72 ± 0.00	8.80 ± 0.00	8.81 ± 0.00
	2.5+MP	8.72 ± 0.01	8.76 ± 0.01	8.74 ± 0.00	8.81 ± 0.01	8.81 ± 0.01
	5+MP	8.72 ± 0.01	8.74 ± 0.00	8.72 ± 0.01	8.80 ± 0.01	8.80 ± 0.00
	10+MP	8.74 ± 0.00	8.77 ± 0.01	8.72 ± 0.01	8.80 ± 0.01	8.80 ± 0.02
O_2 dissolved (mg/l)	**Control**	7.88 ± 0.06	8.18 ± 0.09	9.28 ± 0.07	8.28 ± 0.03	8.40 ± 0.02
	MP	7.93 ± 0.07	8.24 ± 0.01	9.31 ± 0.06	8.27 ± 0.02	8.35 ± 0.02
	1.25	8.06 ± 0.06	8.36 ± 0.02	9.48 ± 0.02	8.48 ± 0.02	8.49 ± 0.02
	2.5	8.02 ± 0.04	8.43 ± 0.00	9.37 ± 0.02	8.49 ± 0.07	8.57 ± 0.01
	5	8.04 ± 0.06	9.45 ± 0.02	9.43 ± 0.06	8.42 ± 0.04	8.78 ± 0.06
	10	8.13 ± 0.11	8.40 ± 0.01	9.59 ± 0.02	8.37 ± 0.02	9.01 ± 0.04
	1.25+MP	8.23 ± 0.09	9.51 ± 0.02	9.34 ± 0.02	8.38 ± 0.02	9.20 ± 0.01
	2.5+MP	8.11 ± 0.06	8.44 ± 0.01	9.45 ± 0.01	8.18 ± 0.01	8.32 ± 0.05
	5+MP	8.17 ± 0.07	8.61 ± 0.05	9.56 ± 0.03	8.39 ± 0.04	8.43 ± 0.07
	10+MP	8.01 ± 0.08	8.39 ± 0.02	9.21 ± 0.03	8.99 ± 0.03	8.79 ± 0.07

Table 16: Nominal and mean actual concentration of cephalexin and cephalexin decay (%) recorded during the bioassay performed at 25ºC. The values are expressed as means ± standard errors of the mean. The S.E.M. are within brackets. Actual cephalexin concentrations were calculated from the individual readings using the linear model: actual cephalexin concentration (mg/l) = - 0.015 + 46.521 x O.D. Deviation (%) relatively to nominal concentrations = 100 – (actual concentration x 100 / nominal concentration). Decay (%) = (mean 0 h absorbance - mean 96 h absorbance) x 100 / mean 0 h absorbance. N – Number of samples analyzed. O.D. – optical density units.

Nominal cephalexin concentrations (mg/l)	**N**	**0h mean (±S.E.M) absorbance readings (O.D.)**	**Mean (±S.E.M) actual cephalexin concentrations (mg/l)**	**Mean deviation (%)**	**96h mean (±S.E.M) absorbance readings (O.D.)**	**Decay (%)**
1.25	8	0.031 (± 0.002)	1.44 (± 0.092)	15.2	0.018 (± 0.002)	44
2.5	8	0.060 (± 0.003)	2.79 (± 0.125)	11.7	0.043 (± 0.004)	30
5	8	0.109 (± 0.005)	5.08 (± 0.225)	1.51	0.094 (± 0.003)	14
10	8	0.198 (± 0.001)	9.21 (± 0.069)	7.91	0.186 (± 0.004)	6
1.25+MP	12	0.032 (± 0.003)	1.47 (± 0.121)	17.4	0.014 (± 0.002)	57
2.5+MP	12	0.060 (± 0.004)	2.78 (± 0.197)	11.4	0.040 (± 0.004)	33
5+MP	11	0.102 (± 0.002)	4.75 (± 0.099)	4.99	0.094 (± 0.002)	8
10+MP	9	0.207 (± 0.001)	9.61 (± 0.064)	3.94	0.184 (± 0.010)	11

Table 17: Nominal and mean actual concentration of MP and MP decay (%) recorded during the bioassay performed at 25ºC. The values are expressed as means ± standard errors of the mean. The S.E.M. are within brackets. Actual MP concentrations were calculated from the individual fluorescence readings using the linear model: actual MP concentration (mg/l) = - 0.1295 + 0.013 x fluorescence (F units). Deviation (%) relatively to nominal concentrations = 100 – (actual MP concentration x 100 /0.184); % Decay = (mean 0 h absorbance - mean 96 h absorbance) x 100 / mean 0 h absorbance. Conc. – concentrations. N – Number of samples analyzed. Nominal concentrations were 0.184 mg/l.

Nominal cephalexin conc. (mg/l)	Nominal MP conc. (mg/l)	N	0h mean (± S.E.M.) fluorescence readings (F units)	Mean (± S.E.M.) actual MP conc. (mg/l)	Deviation (%)	96h mean (± S.E.M.) fluorescence readings (F units)	MP decay (%)
0	0.184	8	25.66 (±0.769)	0.214 (±0.010)	16	14.37 (±0.849)	44
1.25	0.184	12	25.86 (±1.277)	0.217 (±0.017)	18	19.96 (±1.930)	23
2.5	0.184	12	27.80 (±0.443)	0.242 (±0.006)	32	21.42 (±1.641)	23
5	0.184	11	27.47 (±0.802)	0.239 (±0.011)	30	21.72 (±3.085)	21
10	0.184	9	26.32 (±0.852)	0.223 (±0.011)	21	13.91 (±1.088)	47

The actual concentrations of cephalexin in test media at the beginning of the bioassay, estimated from the linear model of Figure 6 from the absorbance of test media are shown in Table 16. In all treatments, the % of deviation of actual concentrations relatively to nominal ones was lower than 20%. Thus, according the OECD guideline nº 203 (OECD, 1992), further results can be expressed in function of nominal concentrations. The decay of cephalexin alone vary between 6% at 10 mg/l and 44% at 1.25 mg/l. The corresponding decay in the presence of MP was 11% and 57%. These findings suggest binding of cephalexin to MP at 25ºC (that was not observed at 20ºC). The decay of MP at

the highest concentration tested was higher at 20ºC than at 25ºC. It was calculated the actual MP concentrations from the linear model of Figure 9 and are shown in Table 17. Mean deviations at 0 h from the nominal concentration (0.184 mg/l) were higher than 20% at 2.5, 5 and 10 mg/l of cephalexin. In these particular cases, the biological results are expressed relatively to the mean actual concentration (OECD, 1992). At 96 h, in all treatments, the decay of MP during the bioassay was higher than 20%, recording an increase of the decay relatively to the decays observed at 20ºC. As in the bioassay performed at 20ºC, such values can be due to the fact that MP may aggregate and precipitate in the test media, not excluding other possibilities.

The total length and weight of the fish measured at the end of the exposure period are indicated in Table 18. The overall mean and the standard error of the mean (S.E.M.) of the total length and weight were 2.6 ± 0.440 cm and 0.216 ± 0.108 g, respectively. For both parameters, no significant differences among treatments were found (total length: $F_{9,85}$= 2.327, p > 0.05; total weight: $F_{9,85}$= 1.152, p > 0.05).

Table 18: Total length and weight of the fish measured after the 96 h bioassay. Results are expressed as the mean of eight to twelve fish per treatment with the respective standard error of the mean.

	Treatment									
	Control	**1.25**	**2.5**	**5**	**10**	**MP**	**1.25+MP**	**2.5+MP**	**5+MP**	**10+MP**
Length (cm)	2.3 ± 0.398	2.5 ± 0.495	2.9 ± 0.423	2.8 ± 0.355	2.6 ± 0.256	2.6 ± 0.381	2.7 ± 0.447	2.6 ± 0.395	2.6 ± 0.552	2.3 ± 0.328
Weight (g)	0.139 ± 0.068	0.222 ± 0.131	0.257 ± 0.118	0.274 ± 0.108	0.182 ± 0.084	0.221 ± 0.097	0.234 ± 0.098	0.206 ± 0.102	0.235 ± 0.124	0.144 ± 0.075

Table 19: Mortality (%) recorded in different treatments after 96 h of exposure to cephalexin alone and in the presence of MP at 25ºC. MP – microplastics present in test media (0.184 mg/l).

	Treatments (mg/l)									
	Control	**MP only**	**1.25**	**2.5**	**5**	**10**	**1.25+MP**	**2.5+MP**	**5+MP**	**10+MP**
Mortality (%)	8	33	33	33	33	33	0	0	8	25

Table 20: Summary of the statistical results obtained on the 2-ANOVA and the Tukey test ($p \leq 0.05$). Pred. perf. - Predatory performance (%). AChE – acetylcholinesterase activity (U/ mg protein). EROD - ethoxyresorufin-O-deethylase activity (U/ mg protein). GST – glutathione-S-transferases activity (U/ mg protein). LPO – lipid peroxidation levels (U/ mg protein). MP – presence of microplastics in test media (0.184 mg/l). CEP – cephalexin. When applied, different letters indicate statistically significant differences. *df* – degrees of freedom; Sig. – level of significance. S.E.M. – standard error of the mean.

Endpoint	Factor	Conc. (mg/l)	Mean ± S.E.M	Tukey test	F	p
Pred. perf.	Treatment				$F_{5, 85} = 19.602$	0.000
		Control	28.18 % ± 1.76	a		
		MP 0.184	20.42 % ± 2.83	b		
		CEP 1.25	21.33 % ± 0.89	a,b		
		CEP 2.5	18.50 % ± 1.42	b.c		
		CEP 5	13.16 % ± 0.82	c,d		
		CEP 10	9.02 % ± 1.59	d		
	MP	0	17.98 % ± 1.41		$F_{1, 85} = 1.872$	0.175
		0.184	17.31 % ± 0.93			
	Interaction				$F_{3, 85} = 0.281$	0.839
AChE	Treatment				$F_{5, 85} = 9.498$	0.000
		Control	95.05 U ± 3.89	a		
		MP 0.184	82.42 U ± 5.67	b		
		CEP 1.25	64.54 U ± 7.80	b		
		CEP 2.5	90.07 U ± 5.63	a		
		CEP 5	88.94 U ± 3.78	a		
		CEP 10	103.6 U ± 5.15	a		
	MP	0	82.07 U ± 2.73		$F_{1, 85} = 10.641$	0.002
		0.184	90.76 U ± 2.73			
	Interaction				$F_{3, 85} = 6.050$	0.001

Continuation Table 20

EROD	Treatment	Control	0.007 U ± 0.002	a,b	$F_{5, 15} = 5.734$	0.004
		MP 0.184	0.005 U ± 0.003	a		
		CEP 1.25	0.017 U ± 0.008	a,b,c		
		CEP 2.5	0.041 U ± 0.014	a,b,c		
		CEP 5	0.054 U ± 0.024	b,c		
		CEP 10	0.081 U ± 0.010	c		
	MP	0	0.025 U ± 0.007		$F_{1, 15} = 2.473$	0.137
		0.184	0.052 U ± 0.012			
	Interaction				$F_{3, 15} = 0.173$	0.913
GST	Treatment	Control	19.13 U ± 2.35		$F_{5, 85} = 0.735$	0.599
		MP 0.184	17.95 U ± 2.22			
		CEP 1.25	22.79 U ± 1.84			
		CEP 2.5	19.32 U ± 2.04			
		CEP 5	21.14 U ± 1.94			
		CEP 10	20.48 U ± 2.13			
	MP	0	19.21 U ± 1.27		$F_{1, 85} = 2.652$	0.107
		0.184	21.53 U ± 1.16			
	Interaction				$F_{3, 85} = 0.659$	0.579

Continuation Table 20

LPO	Treatment			$F_{5, 85}$ = 1.332	0.259
		Control	0.46 U ± 0.08		
		MP 0.184	0.34 U ± 0.08		
		CEP 1.25	0.63 U ± 0.18		
		CEP 2.5	0.56 U ± 0.07		
		CEP 5	0.46 U ± 0.04		
		CEP 10	0.67 U ± 0.07		
	MP	0	0.45 U ± 0.04	$F_{1, 85}$ = 1.616	0.207
		0.184	0.49 U ± 0.06		
	Interaction			$F_{3, 85}$ = 3.176	0.028

The results of cephalexin in the presence and absence of MP at 25ºC on distinct parameters of fish are shown in Figures 16 and 17, and the results of the 2-ANOVA are shown in Table 20. Concerning the predatory performance, significant differences among treatments, no significant differences induced by the presence of MP, and no significant interaction between treatments and MP presence were found (Table 20). Considering the overall means per treatment, cephalexin induced effects significantly different from the control group at concentrations equal or higher than 2.5 mg/l reaching 32% of predatory performance inhibition at the highest concentration tested. MP alone induced significant predatory performance differences relatively to the control group. The no significant interaction suggests that, at 25ºC the presence of MP did not interact with the effects of cephalexin on fish predatory performance.

Regarding AChE activity, significant differences among treatments, significant differences induced by the presence of MP and a significant interaction between treatments and the presence of MP were found (Table 20). Considering the overall means per treatment, cephalexin induced effects significantly different from the control group at 1.25 mg/l (32% of inhibition) and on fish only exposed to MP (14% of inhibition). Since there were differences in the presence and absence of MP, all the treatments were compared with 1-ANOVA ($F_{9, 85}$ = 5.651) and the Tukey test (Figure 15). The significant interaction observed here indicate that the presence of MP negatively affect the effects of cephalexin on fish AChE activity.

Concerning EROD activity, results of the 2-ANOVA suggests significant differences among treatments, whereas no significant differences induced by the presence of MP and no significant interaction between the two previous factors were found (Table 20). Although results are not significant, there is a tendency for the increase of EROD activity in the presence of MP and as the concentration of cephalexin increases, where EROD activity showed to be 10 folds higher at the highest concentration tested in the presence of MP, compared to the control group. MP alone did not induce significant differences relatively to the control group.

With regard to GST activity, no significant differences among treatments, no significant differences induced by the presence of MP and no significant interaction between treatments and the presence of MP were found (Table 20). Besides, no significant effects were observed in fish only exposed to MP.

For LPO levels, results of the 2-ANOVA showed no significant differences among treatments, no significant differences induced by the presence of MP and significant differences between treatments and the presence of MP (Table 20). These findings suggest that although there is an interaction between the factors, the results obtained do not allow to sustain that a scenario of oxidative stress has been installed after the exposure of the *P.microps* to cephalexin alone and combined to MP at 25ºC, in the context of the concentrations tested.

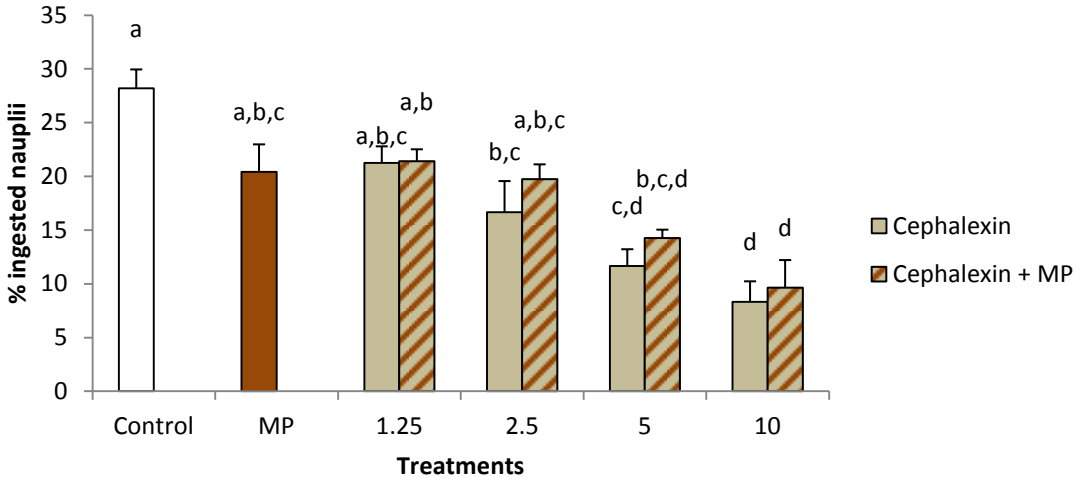
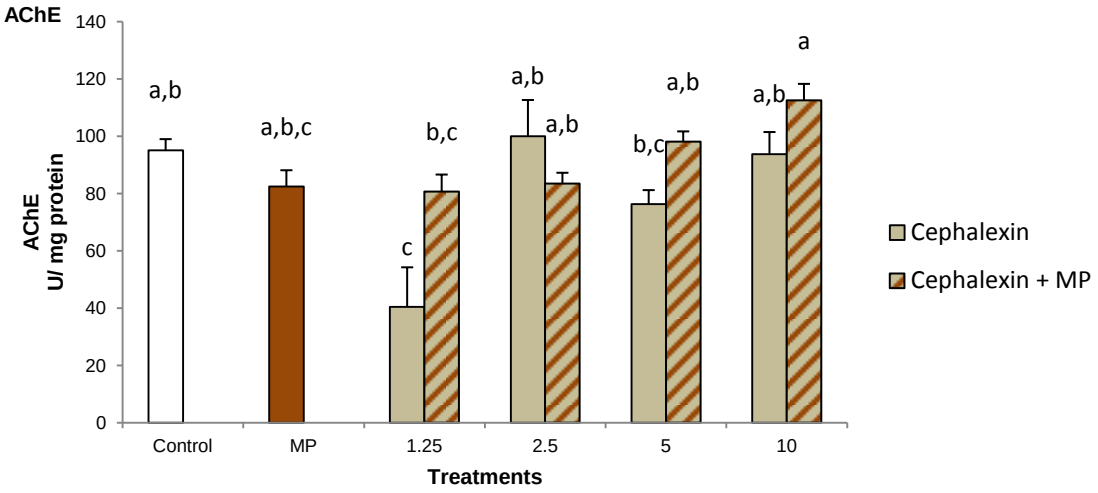
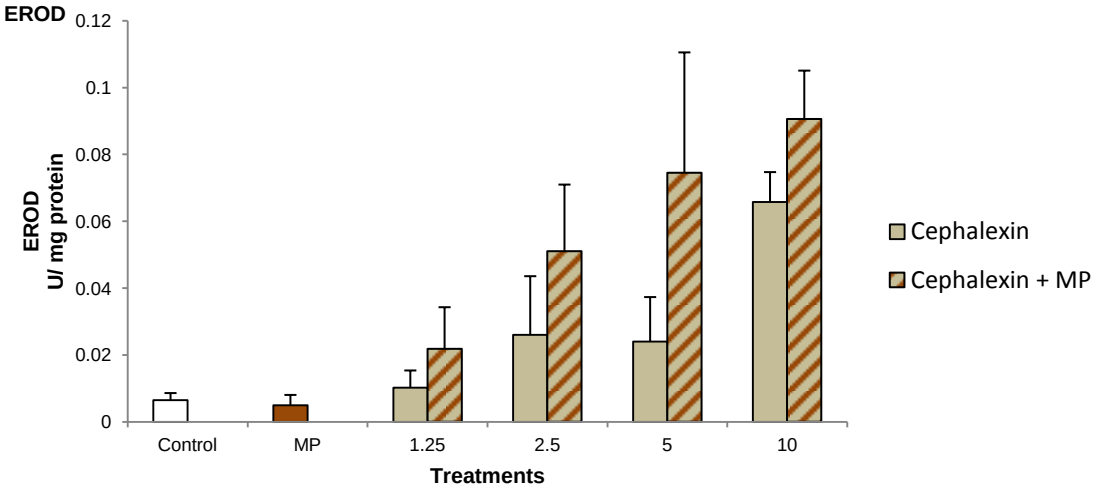
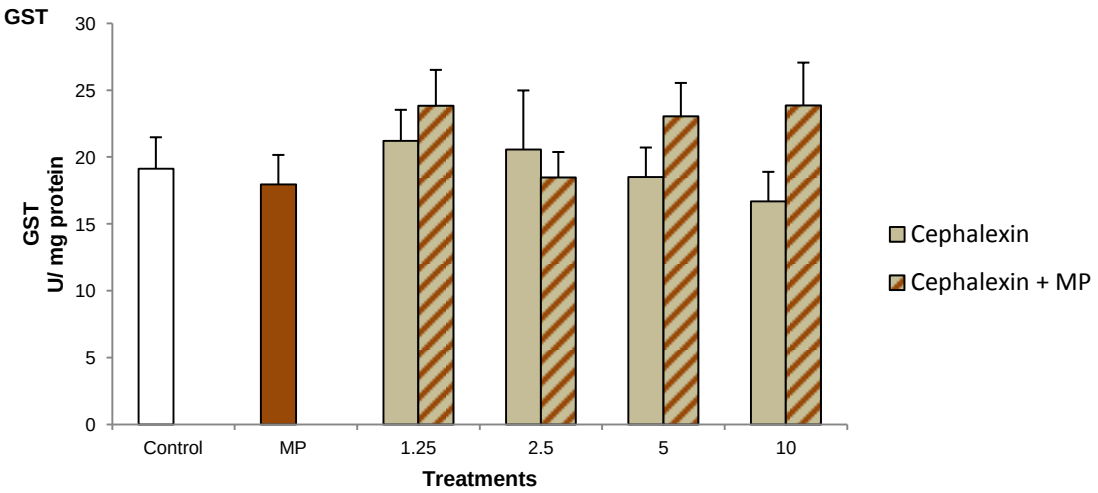


Figure 16: Predatory performance of *Pomatoschistus microps* assessed individually after 96 h of exposure to cephalexin alone (1.25, 2.5, 5 and 10 mg/l) and combined to microplastics (0.184 mg/l) and to microplastics alone (0.184 mg/l). Eight to twelve fish were used per treatment. Results are the mean of the percentage of ingested nauplii relatively to the total number offered (30) with corresponding S.E.M bars.


AChE
AChE U/ mg protein
140
120
100
80
60
40
20
0
a,b
a,b,c
b,c
c
a,b
a,b
b,c
a,b
a
Control
MP
1.25
2.5
5
10
Treatments
Cephalexin
Cephalexin + MP


EROD
EROD U/ mg protein
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
MP
1.25
2.5
5
10
Treatments
Cephalexin
Cephalexin + MP


GST
GST U/ mg protein
30
25
20
15
10
5
0
Control
MP
1.25
2.5
5
10
Treatments
Cephalexin
Cephalexin + MP

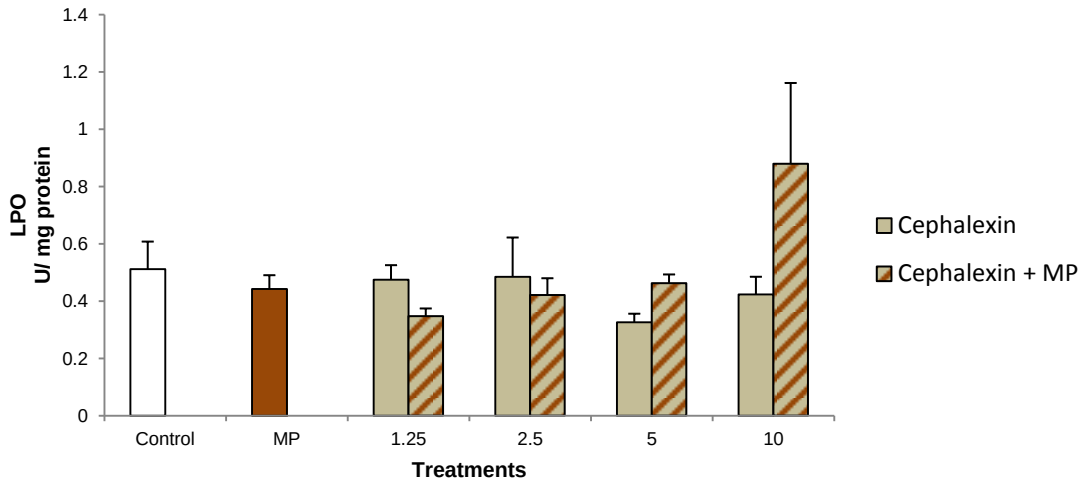


Figure 17: Effects of cephalexin alone (1.25, 2.5, 5 and 10 mg/l) and in combination of microplastics and effects of microplastics alone on the activity of acetylcholinesterase (AChE), ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferases (GST) enzymes and lipid peroxidation levels (LPO) of *Pomatoschistus microps* juveniles after 96 h of exposure. Different letters above the bars indicate statistically significant differences (1-ANOVA and Tukey's multi-comparison test). The results are the mean of eight to twelve fish with corresponding standard error bars. U – nmol/min for AChE and GST activity, pmol/min for EROD activity and nmol of TBARS/min for LPO levels.

3.7. Influence of temperature on the chemicals toxicity

With the aim to understand if a raise of 5ºC in temperature cause negative impacts on the endpoints tested, a 2-ANOVA was performed. Results are shown in Table 21.

Table 21: Summary of the statistical results obtained on the 2-ANOVA and the Tukey test ($p \leq 0.05$). Pred. perf. - Predatory performance (%). AChE – acetylcholinesterase activity (U/ mg protein). EROD - ethoxyresorufin-O-deethylase activity (U/ mg protein). GST – glutathione-S-transferases activity (U/ mg protein). LPO – lipid peroxidation levels (U/ mg protein). MP – presence of microplastics in test media (0.184 mg/l). CEP – cephalexin. When applied, different letters indicate statistically significant differences. df – degrees of freedom; Sig. – level of significance. S.E.M. – standard error of the mean.

Endpoint	Factor	Conc. (mg/l)	Mean ± S.E.M	Tukey test	F	p
Pred. perf.	Treatment				$F_{9, 195} = 11.350$	0.000
		Control	31.67% ± 1.74	a		
		MP 0.184	24.21% ± 2.78	a,b		
		CEP 1.25	24.83% ± 1.63	a,b		
		CEP 1.25 + MP	20.28% ± 1.39	b,c		
		CEP 2.5	21.17% ± 2.32	b,c		
		CEP 2.5 + MP	19.31% ± 1.17	b,c		
		CEP 5	17.41% ± 1.94	b,c,d		
		CEP 5 + MP	17.42% ± 1.45	b,c,d		
		CEP 10	12.46% ± 1.63	d		
		CEP 10 + MP	14.74% ± 2.00	c,d		
	Temperature				$F_{1, 195} = 29.860$	0.000
		20ºC	22.94% ± 0.97			
		25ºC	17.61% ± 0.81			
	Interaction				$F_{9, 195} = 2.108$	0.031

Continuation Table 21

AChE	Treatment	Control	76.95 U ± 4.17	a,b	$F_{9, 195} = 11.315$	0.000
		MP 0.184	69.49 U ± 4.54	a		
		CEP 1.25	44.22 U ± 4.46	d		
		CEP 1.25 + MP	75.64 U ± 3.99	a,b		
		CEP 2.5	81.15 U ± 4.46	a,b		
		CEP 2.5 + MP	81.30 U ± 3.99	a,b,c		
		CEP 5	66.09 U ± 4.63	a		
		CEP 5 + MP	91.58 U ± 4.17	b,c		
		CEP 10	76.39 U ± 4.54	a,b		
		CEP 10 + MP	99.50 U ± 4.49	c		
	Temperature	20ºC	66.21 U ± 1.85		$F_{1, 195} = 53.155$	0.000
		25ºC	86.26 U ± 2.03			
	Interaction				$F_{9, 195} = 2.990$	0.002
EROD	Treatment	Control	0.028 U ± 0.01		$F_{9, 34} = 3.332$	0.005
		MP 0.184	0.027 U ± 0.01			
		CEP 1.25	0.012 U ± 0.02			
		CEP 1.25 + MP	0.040 U ± 0.01			
		CEP 2.5	0.029 U ± 0.01			
		CEP 2.5 + MP	0.052 U ± 0.01			
		CEP 5	0.041 U ± 0.01			
		CEP 5 + MP	0.078 U ± 0.01			
		CEP 10	0.041 U ± 0.01			
		CEP 10 + MP	0.067 U ± 0.01			
	Temperature	20ºC	0.045 U ± 0.01		$F_{1, 34} = 6.614$	0.015
		25ºC	0.038 U ± 0.01			
	Interaction				$F_{9, 34} = 2.872$	0.012

Continuation Table 21

GST	Treatment	Control	13.25 U ± 1.50	a,b	$F_{9, 195} = 8.118$	0.000
		MP 0.184	13.90 U ± 1.63	a,b		
		CEP 1.25	15.03 U ± 1.60	a,b,c		
		CEP 1.25 + MP	22.80 U ± 1.43	d,e		
		CEP 2.5	13.99 U ± 1.60	a,b		
		CEP 2.5 + MP	19.11 U ± 1.43	b,c,d,e		
		CEP 5	11.45 U ± 1.66	a		
		CEP 5 + MP	24.27 U ± 1.50	e		
		CEP 10	16.63 U ± 1.63	a,b,c,d		
		CEP 10 + MP	20.71 U ± 1.61	c,d,e		
	Temperature	20ºC	13.97 U ± 0.83		$F_{1, 195} = 42.412$	0.000
		25ºC	20.48 U ± 0.85			
	Interaction				$F_{9, 195} = 4.282$	0.000
LPO	Treatment	Control	0.484 U ± 0.09		$F_{9, 195} = 3.269$	0.001
		MP 0.184	0.392 U ± 0.10			
		CEP 1.25	0.589 U ± 0.10			
		CEP 1.25 + MP	0.448 U ± 0.09			
		CEP 2.5	0.404 U ± 0.10			
		CEP 2.5 + MP	0.608 U ± 0.09			
		CEP 5	0.354 U ± 0.10			
		CEP 5 + MP	0.499 U ± 0.09			
		CEP 10	0.523 U ± 0.10			
		CEP 10 + MP	0.801 U ± 0.10			
	Temperature	20ºC	0.543 U ± 0.04		$F_{1, 195} = 0.704$	0.403
		25ºC	0.477 U ± 0.04			
	Interaction				$F_{9, 195} = 2.328$	0.017

At 20ºC, the decay of cephalexin was less than 20% either in the absence as in the presence of MP. At 25ºC, the decay of the substance was higher than 20% both in the absence and presence of MP in the two lowest concentrations tested (1.25 an 2.5 mg/l).

At 20ºC and 25ºC, mean deviations from nominal concentrations were always lower than 20%, so the biological results were expressed relatively to the nominal concentrations. With regard to the decay of MP, all treatments showed decay higher than 20% both at 20ºC and 25ºC, but at 25ºC, this decay showed to be greater. This suggests that MP may aggregate and precipitate, not excluding other possibilities. Regarding the mean deviations of MP from nominal concentrations, at 20ºC, the deviation was less than 20% at 20ºC, whereas at 25ºC, the mean deviation showed to be higher than 20% at the three highest concentrations tested. At 25ºC, the predatory performance and AChE activity of fish only exposed to MP were significantly different from the control group. At 20ºC, such thing did not happen.

The results of cephalexin at the two distinct temperature on distinct parameters of fish are shown in Figures 18 and 19, and the results of the 2-ANOVA are shown in Table 21. Concerning the predatory performance, significant differences among treatments, significant differences induced by the increased temperature and significant interaction between treatments and temperature were found (Table 21). Considering the overall means per treatment, cephalexin induced effects significantly different from the control group in all the treatments of cephalexin alone and in combination with MP, except for the lowest cephalexin concentration alone, reaching 61% and 53% of predatory performance inhibition at the highest concentration tested alone and combined to MP, respectively. The comparison of the predatory performance of fish exposed to different temperatures per treatment (Student t-test, Figure 18), indicates significant lower predatory performance at 25ºC in fish of the control group and in fish exposed to the two highest cephalexin concentrations both in the absence and presence of MP than at 20ºC. These results suggest that an increase of temperature negatively affect the effects of the tested substances and is in good agreement with the significant interaction between treatment and temperature indicated by the two-way ANOVA (Table 21). These findings indicate that fish may suffer because of the low quantity of food ingested, potentially resulting weakness, which may leads to a reduced growth capacity, reduced escape from predators, reduced reproductive capacity and an increased probability of death. The decrease of the predatory performance with the temperature raise is in agreement with findings from studies in the literature, indicating that high temperatures cause changes in the predatory performance. For example, in a study with *Daphnia pulex* as predator and phytoplankton as prey, the higher temperature (25ºC) destabilized the predator-prey system compared to what happened at the lowest temperature (18ºC) (Beisner *et al.,* 1997). However, the results of the present study are not in agreement with the results obtained by Eck *et al.,* (2014), where they found that two dragonfly larvae, *Aeshna*

interrupta and *Didymops transversa* showed a modest increase in predation in the warmest temperatures (24.5ºC and 28ºC comparatively to 16.6ºC and 20ºC).

Regarding AChE activity, significant differences among treatments, significant differences induced by the increased temperature and significant interaction between treatments and temperature were found (Table 21). The lowest cephalexin concentration alone and the highest cephalexin concentration in the presence of MP were the only treatments significantly different from the control group (Table 21). Since there were differences in fish exposed to distinct temperatures, all the treatments at 20ºC were compared to the corresponding treatment at 25ºC (Student t-test, Figure 19). The results indicate significant higher AChE activity at 25ºC in fish of the control group, in fish exposed to MP alone, to 2.5 mg/l of cephalexin alone and to the two highest cephalexin concentrations alone and combined to MP than at 20ºC. The significant interaction indicates that temperature increase interact with the effects of substances on fish AChE activity. The increase of fish AChE activity with the temperature raise observed in all the treatments including the control group, is not in agreement with findings from Scaps & Borot (2000), where they found that AChE activity of the Sandworm (*Nereis diversicolor*) tended to decrease as temperature increase.

For EROD activity, significant differences among treatments, significant differences induced by the increased temperature and significant interaction between treatments and temperature were found (Table 21). Because differences in fish exposed to distinct temperature were observed, all the treatments at 20ºC were compared to the corresponding treatment at 25ºC (Student t-test, Figure 19). The results indicate significant lower EROD activity at 25ºC in fish of the control group and in fish only exposed to MP than at 20ºC. However, there is variability in the results. This variability may be due to the low enzymatic content of the fractions used. It would have been more appropriate to analyze only the fish livers, but because fish bodies are too small, it would have been necessary a large number of organisms to obtain appropriate samples to determine the EROD activity. Instead, the whole body was used, thus increasing the error and variability of the analyses. The interaction observed indicate that temperature interact with the effects of substances on fish EROD activity, suggesting that the involvement of EROD activity of the biotransformation of cephalexin and MP cannot be despised, being necessary more investigation on this subject.

With respect to GST activity, significant differences among treatments, significant differences induced by the increased temperature and significant interaction between treatments and temperature were found (Table 21). Considering the overall means per treatment, cephalexin induced effects significantly different from the control group at 1.25, 2.5 and 10 mg/l of cephalexin in the presence of MP. Since there were differences in fish

exposed to distinct temperatures, all the treatments at 20ºC were compared to the corresponding treatment at 25ºC (Student t-test, Figure 19). The results indicate significant higher GST activity at 25ºC in fish of the control group, in fish exposed to MP alone, and fish exposed to 1.25, 2.5 and 5 mg/l of cephalexin alone than at 20ºC. These results suggest toxicological interaction between all the treatments and temperature. These findings may be of high interest to the fish fitness in their natural environment due to the crucial role that GST has on the detoxification process of xenobiotics and on the protection against oxidative stress in the context of global climate changes. The increase of fish GST activity with the temperature raise is in agreement with findings from Madeira *et al.,* 2013, where higher GST activity induced by increasing the temperature by 1ºC/h in sea Bass (*Dicentrarchus labrax*), Thin-lip mullet (*Liza ramada*), White seabream (*Diplodus sargus*), and in Common two-banded seabream (*Diplodus vulgaris*) were observed.

Relatively to LPO levels, 2-ANOVA showed significant differences between treatments but not between temperatures and significant interaction between the two previous factors (Table 21). The comparison of the LPO levels of fish exposed to different temperatures per treatment (Student t-test, Figure 19), indicates significant lower LPO levels at 25ºC in fish exposed to 1.25 and 2.5 mg/l of cephalexin combined to MP than at 20ºC. The decrease of fish LPO levels at 25ºC in the treatments significantly different at different temperatures can be explained by the increase of GST activity at 25ºC. However, there is variability in the results, so its interpretation needs to be careful. The decrease of fish LPO levels with the temperature raise observed in the significant treatments is in agreement with findings from Vinagre *et al.,* (2012), where they detected lower LPO levels in the sea bass (*Dicentrarchus labrax*) at 24ºC comparatively to fish exposed at 18ºC. However, other studies obtained different results. For example, in a study using the thorn fish (*Terapon jarbua*), fish exposed at 28, 32 and 36ºC obtained higher LPO levels as the temperature increase (Chien & Hwang, 2001). Another study also detected higher LPO levels in response to thermal stress induced by increasing the temperature by 1ºC/h in sea Bass (*D. labrax*), Thin-lip mullet (*L. ramada*), and Sargo (*D. sargus*) (Madeira *et al.,* 2013).

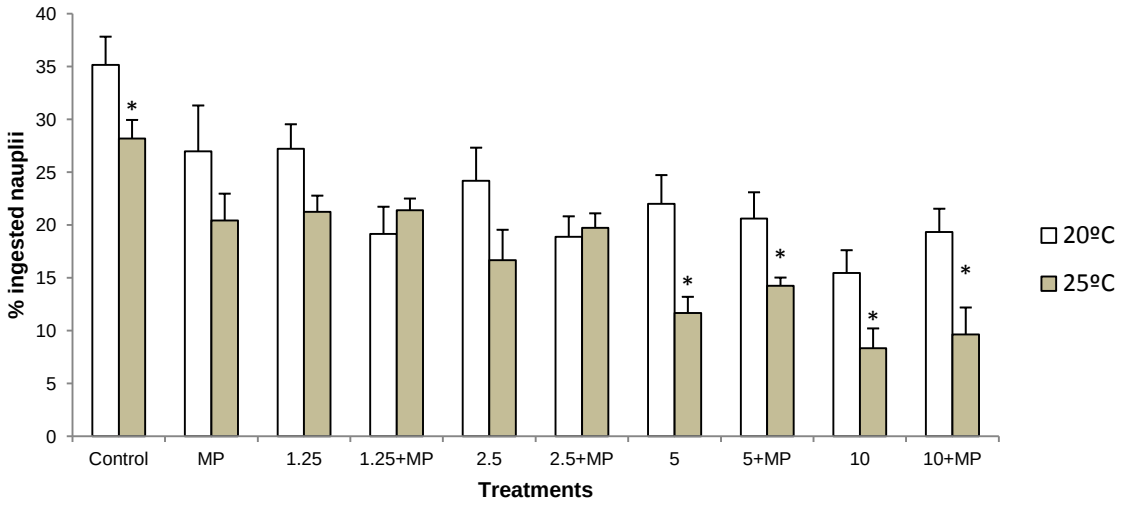
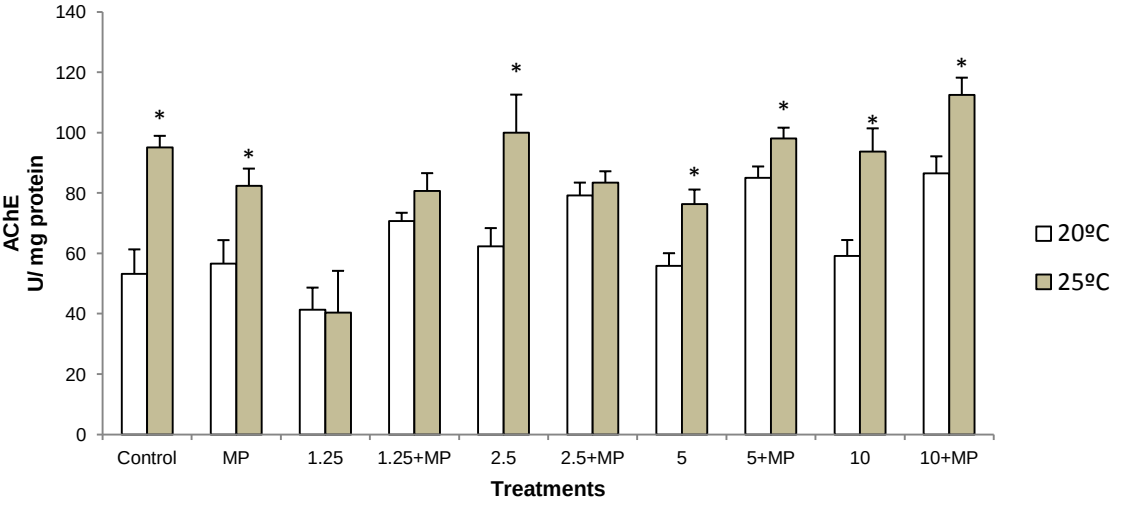
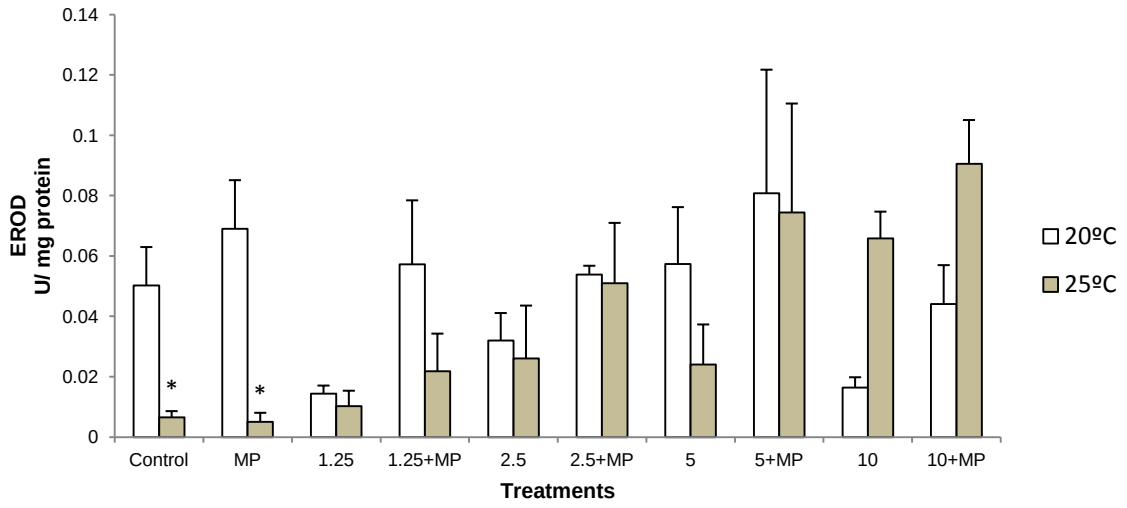
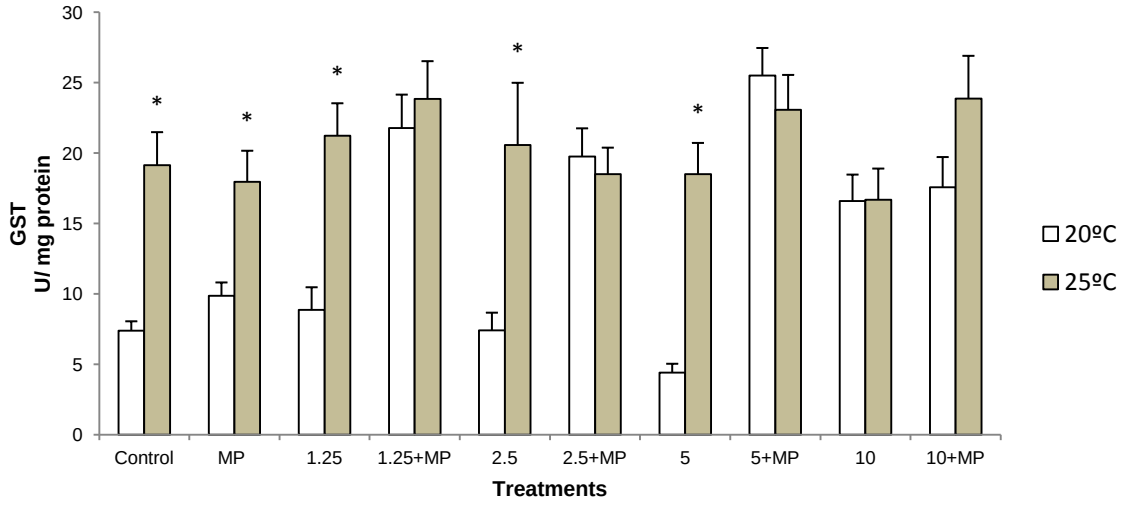


Figure 18: Predatory performance of *Pomatoschistus microps* assessed individually after 96 h of exposure to cephalexin alone (1.25, 2.5, 5 and 10 mg/l) and combined to microplastics (0.184 mg/l) and to microplastics alone (0.184 mg/l). Eight to twelve fish were used per treatment. * - significantly different from the same treatment made at different temperature (Student's t-test, $p < 0.05$). Results are the mean of the percentage of ingested nauplii relatively to the total number offered (30) with corresponding S.E.M bars.




EROD
U/ mg protein
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
MP
1.25
1.25+MP
2.5
2.5+MP
5
5+MP
10
10+MP
Treatments
20ºC
25ºC


GST
U/ mg protein
30
25
20
15
10
5
0
Control
MP
1.25
1.25+MP
2.5
2.5+MP
5
5+MP
10
10+MP
Treatments
20ºC
25ºC

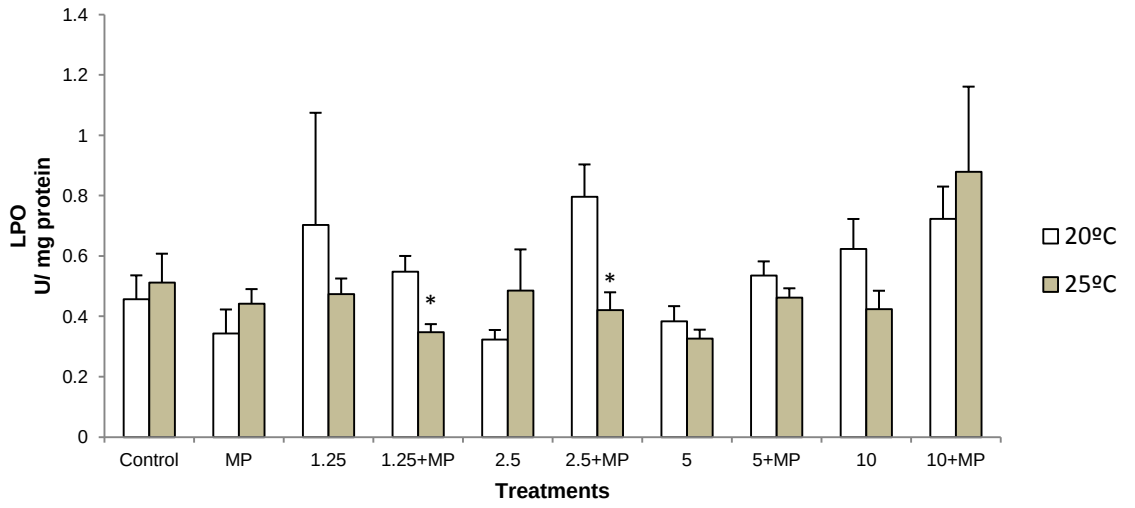


Figure 19: Effects of cephalexin alone (1.25, 2.5, 5 and 10 mg/l) and in combination of microplastics and effects of microplastics alone on the activity of acetylcholinesterase (AChE), ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferases (GST) enzymes and lipid peroxidation levels (LPO) of *Pomatoschistus microps* juveniles after 96 h of exposure. * - significantly different from the same treatment made at different temperature (Student's t-test, $p < 0.05$). The results are the mean of eight to twelve fish with corresponding standard error bars. U – nmol/min for AChE and GST activity, pmol/min for EROD activity and nmol of TBARS/min for LPO levels.

CHAPTER IV

4. Main Conclusions

Nowadays, the contamination of the marine environment by pharmaceuticals and microplastics and the global warming have been increasingly gaining importance due to the negative impacts on human and environmental health.

The spectrophotometric and spectrofluorometric methods used to determine the actual concentrations and decay of cephalexin and MP respectively were validated in the present study for use in ASW and are cost-effective methods. However, it should be noted that the spectrophotometric method may lose precision, as suggest by the very low absorbance values and the high departure from the nominal concentrations observed at the lowest concentrations. Because, in some cases, the decay of both cephalexin and MP was higher than 20%, it is recommended the renewed of the media, at least at 48 h. According to the results obtained, after a short period of time (96 h), cephalexin alone significantly decreased the predatory performance of *P. microps* juveniles at concentrations equal or higher than 2.5 mg/l, induced AChE and GST activity at 1.25 and 10 mg/l, respectively, thus corroborating our first hypothesis (cephalexin is able to induce toxic effects on *P. microps* juveniles at concentrations in the low ppm range). No EROD activity and lipid peroxidation caused by cephalexin was found. At 20ºC, when simultaneously exposed to cephalexin and MP, fish had inhibition of the predatory performance at concentrations equal or higher than 1.25 mg/l. These findings suggest that the reduced ability of the fish to capture a prey may result in weakness, which may leads to a reduced growth capacity, reduced escape from predators, reduced reproductive capacity and an increased probability of death. These potential impacts may decrease the population fitness and thus its ecological function. Due to the cruciality of the predatory performance for the survival of the species, it is of great importance to further investigate the hypothesis that cephalexin, MP and emerging contaminants in general, may have negative effects on this endpoint. The increased AChE activity observed in fish exposed to the contaminants mixture at concentrations equal or higher than 5 mg/l indicate that cephalexin affect the cholinergic function of the fish. Results obtained may explain the lower predatory performance. In fact, high levels of AChE have unpredictable consequences yet but have been associated with problems in muscle function and on neuromuscular transmission in general. Therefore, the increase in AChE activity caused by the administration of certain drugs may lead to reduction cholinergic neurotransmission efficiency due to a decrease in acetylcholine levels in the synaptic cleft (Ferreira et al.,

2012). An induction of the GST activity was observed at concentrations equal or higher than 1.25 mg/l of cephalexin combined to MP. These results corroborate our second hypothesis (the presence of MP influences the toxic effects of cephalexin). At 20ºC, MP alone did not induce significant effects in any of the endpoint tested. However, at 25ºC, the predatory performance and AChE activity of the fish appeared to be significantly different from the control group when exposed to MP only. The comparison of the control groups at 20ºC and 25ºC shows that the increase of temperature changed some of the analyzed parameters. In fact, the Student t-test realized indicates that the control groups of the two distinct temperatures are significantly different from each other for all the parameters tested except for LPO levels. The interaction between treatments and temperature showed to be significant in all the endpoints tested, suggesting that temperature modulates the effects of the tested substances. Significant differences among treatments were found, but the Tukey test was not able to discriminate statistically significant differences for EROD activity and LPO levels. In fact, the results of the 2-ANOVA demonstrated that only the fish predatory performance (inhibition at 1.25, 2.5, 5 and 10 mg/l of cephalexin alone and in combination with MP), AChE activity (induction at 1.25 mg/l of cephalexin alone and 10 mg/l of cephalexin with MP) and GST activity (induction at 1.25, 5 and 10 mg/l of cephalexin with MP) were significantly different from the control group. Besides, the Student's t-test performed to see differences between the same treatment made at the two different temperatures, showed differences in certain cases, indicating different effects from fish exposed at 20 and 25ºC. These findings indicated increased toxicological interactions between the substances and temperature on *P. microps*, thus corroborating our third hypothesis.

Although the concentrations tested are not environmentally relevant, one must take into consideration that organisms are rarely exposed to single substances but instead to mixtures of different compounds with similar modes of action or not. Therefore, the findings of the present study are relevant and allow us to understand potential toxicological interactions in the context of global climate changes.

CHAPTER V

5. List of references

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CHAPTER VI

6. Annex

Table A 1: Physico-chemical parameters monitored in the days catch of *P.microps* in Minho River estuary.

Capture date	Temperature (ºC)	pH	Oxygen dissolved (mg/l)	Salinity (g/l)
November 2013	12.6	7.55	7.9 mg/l – 74.3%	6
February 2014	14	8.22	8.07 mg/l – 77.5%	3
May 2014	24.9	7.85	8.88 mg/l – 106.2%	6
July 2014	22.5	8.55	12.2 mg/l – 140.1%	22

Table A 2: Physico-chemical parameters monitored every 24h during the acclimatization period.

Time	Bioassay	Temperature (ºC)	pH	Oxygen dissolved (mg/l)	Salinity (g/l)
0h	Training bioassay	21.7	8.15	8.19	18
	Preliminary bioassay	21.5	8.30	8.51	18
	Bioassay at 20ºC	21.3	8.48	8.60	18
	Bioassay at 25ºC	23.6	8.09	8.38	18
24h	Training bioassay	21.9	8.15	8.52	19
	Preliminary bioassay	22.3	8.21	8.35	20
	Bioassay at 20ºC	21.2	8.43	8.59	19
	Bioassay at 25ºC	24.0	8.35	8.22	19
48h	Training bioassay	22.0	8.32	8.36	19
	Preliminary bioassay	21.8	8.00	8.15	20
	Bioassay at 20ºC	21.4	8.47	8.56	19
	Bioassay at 25ºC	23.7	8.26	8.35	19
72h	Training bioassay	21.5	8.17	8.43	18
	Preliminary bioassay	22.1	7,85	7.94	20
	Bioassay at 20ºC	21.4	8.43	8.79	18
	Bioassay at 25ºC	23.9	8.28	8.29	18
96h	Training bioassay	22.3	8.26	8.65	19
	Preliminary bioassay	22.2	7.89	8.04	18
	Bioassay at 20ºC	21.4	8.46	8.65	19
	Bioassay at 25ºC	23.8	8.15	8.39	19